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ANTI-ARTHRITIC EFFECT OF 1'-ACETOXYCHAVICOL ACETATE IN COMPLETE FREUNDS ADJUVANT-INDUCED ARTHRITIS IN RATS.

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ABSTRACT

1'-Acetoxychavicol acetate (ACA) is a phenylpropanoid present in *Alpinia galanga* (L) Willd syn. *Languas galanga*. The objective of this study was to isolate 1'-Acetoxychavicol acetate (ACA) from acetone extract of *Alpinia galanga* (AEAG) and evaluate anti-arthritic activity of 1'-Acetoxychavicol acetate (ACA) and acetone extract of *Alpinia galanga* (AEAG) on complete Freund's Adjuvant-induced arthritis in rats. Radiographic and histopathological observations were evaluated. AEAG (400 mg/kg) and ACA (10 and 20 mg/kg) suppressed the paw swelling, increased the paw withdrawal latency, and reduced the paw thickness in arthritic rats. Radiological and histopathological characteristics in ACA (20 mg/kg) treated rats were comparable with that of diclofenac treated rats. The present study indicates that ACA and AEAG showed significant antiarthritic activity.

KEYWORDS : 1'-Acetoxychavicol acetate, Anti-arthritic, Acetone extract of *Alpinia galanga,* Complete Freund's adjuvant.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease affecting about 1% of the population in developed countries[1]. Although the disease can start at any age, the peak onset is between 25 and 55 years, women being affected about three times more frequently than men[2]. It is characterized by progressive joint destruction, deformity, disability and premature death in most patients[3]. Medication like non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and analgesics are used to suppress the symptoms but they may cause side effects[4]. NSAIDs help to relieve pain and stiffness and reduce inflammation; however, they do not slow down the progression of rheumatoid arthritis[5]. It seems for that reason of relevance to develop new strategies for treating pain in muscle and joints, therefore need to develop medication that should be affordable and associated with a minimum of side effects[6]. However, chronic use of NSAIDs is associated with gastrointestinal toxicity, which has limited their use and leads to the search for safer alternative agents. *A. galanga* (L) Willd syn. *Languas galanga* commonly called greater Galangal (Zingiberaceae) is distributed in various parts of India and Southeast Asia. It is used as food additive in Thailand and other parts of Asia. The rhizomes were used for treatment of rheumatism, bronchial catarrh, bad breath, ulcers, whooping cough, throat infections, incontinence, fever and microbial infections, headache, rheumatic pain, and pain in chest[7, 8]. *A. galanga* contains various secondary metabolites like, terpenoids, flavonoids, phenylpropanoids etc. One such medicinally important compound present in the rhizomes of this plant is ACA. ACA is a phenylpropanoid, responsible for anti-inflammatory, anti-plasmid, and inhibition of HIV-1 by blocking rev transport[9-12]. Chavicol analogues from *A. galanga* have been shown to have potent antioxidant and anti-inflammatory activities[13]. In addition ACA inhibited RANKL–induced osteoclastic differentiation of RAW 264.7 monocytic cells by suppressing nuclear factor-κB activation[14]. It possesses potent inhibitory effect on the production of nitric oxide (NO) in lipopolysaccharide-activated mouse peritoneal macrophages as well as non-specific COX inhibition activity[15, 16]. However, anti-arthritic activity of ACA has not been reported in CFA induced arthritis. The objective of the study was to evaluate anti-arthritic activity of ACA and AEAG.

MATERIALS AND METHODS

(i) Plant materials

The rhizomes of *A. galanga* were purchased from local market in Pune, in August 2012 and were identified by Dr. A.S.Upadhya, Scientist, Plant drug authentication service (Botany group) plant science division, Agharkar Research Institute, Pune, Maharashtra, India. The voucher specimen (R-167) is deposited in the same institute.

(ii) Animals

Healthy female wistar rats (150-200 g) and healthy female Swiss albino mice (20-25 g) were procured from National Institute of Biosciences, Pune. Animals were housed at 24 ± 1°C and relative humidity of 65 ± 10% and at standard environmental conditions (12 h light and 12 h dark cycle) in the animal house of the college. The animals were fed with standard pellet rodent diet and water was provided *ad libitum*. All the experimental protocols used in this study were approved by Institutional Animal Ethical Committee.

(iii) Preparation of acetone extract of rhizomes of A. galanga and isolation of 1'ACA[11]

The rhizomes were shade dried; cut in small pieces and powdered in hand mixer. The dried powder of the rhizomes of *A. galanga* (500 g) was extracted with 1.5L acetone as a solvent by cold percolation for 12 h in a 5L flat bottom flask at room temperature. The process of extraction was repeated three times with acetone. Each time the filtrate was concentrated *in vacuo* at 40°C using a rotary evaporator (Eqitron, Roteva), and pooled together to obtain 18.60 g of reddish extract.

The crude acetone extract (10.0 g) was packed in silica-gel column with (60-120 mesh). The material was eluted stepwise with a gradient of n-hexane-acetone. The fractions were collected separately and concentrated *in vacuo* at 40°C. These fractions in the TLC were pooled together to obtain 5 fractions: A (3.23 g), B (2.88 g), C (1.92g), D (0.55 g) and E (0.25 g). The fraction B (2.50 g) was repacked in silica gel column. The column was eluted with gradient of hexane and acetone. The fractions obtained were concentrated and identified by TLC and similar fractions were pooled together to obtain sub-fraction B_1 (0.23 g), B_2 (1.65 g), B_3 (0.45 g) and B_4 (0.25 g). The sub-fraction B_2 (1.60 g) was separated by preparative TLC [n-hexane:ethylacetate (8:2)] to yield compound (1), as a yellow oil (yield 1,20 g), identification of which was performed by comparison of the spectral data of mass and nuclear magnetic resonance with that of reported spectral data in literature. All spectral data were obtained on the following instruments; IR were recorded on a Perkin-Elmer model (683 B) and absorption is expressed in cm^{-1}. NMR on (Brucker AC-200) spectrometer. GC-MS on a (Clarus 500) spectrometer. Optical rotation was measured using digital polarimeter (JASCO-181).

(iv) Acute oral toxicity study[17]

Healthy female Swiss albino mice were subjected to acute toxicity studies as per OECD guideline-425. The animals were fasted overnight and divided into groups of 5 animals. Acetone extract of *A. galanga* (AEAG) was administered orally at one dose level of 2000 mg/kg body weight. All mice were observed for 48 hrs for any sign of toxicity or mortality.

(v) Complete Freund's adjuvant (CFA) induced arthritis in rats[18]

Arthritis was induced by the intradermal injection of 0.1 ml of Complete Freund's Adjuvant (CFA, Sigma) in the right hind paw of female wistar rats of 150 to 200 g body weight. The animals were divided into nine groups of six animals each as follows: Group 1- Non-arthritic, group 2- Arthritic control, group 3- Arthritic animals treated with standard, diclofenac 5 mg/kg, p.o, group 4- Arthritic animals treated with test extract, AEAG 100 mg/kg, p.o, group 5- Arthritic animals treated with test extract, AEAG 200 mg/kg, p.o, group 6- Arthritic animals treated with test extract, AEAG 400 mg/kg, p.o, group 7- Arthritic animals treated with test compound, ACA 5 mg/kg, p.o, group 8- Arthritic animals treated with test compound, ACA 10 mg/kg, p.o, group 9- Arthritic animals treated with test compound, ACA 20 mg/kg, p.o. The dosing of all the groups was started from 12th day post CFA injection, once day. The following parameters were measured at regular intervals (Day 0, 1, 4, 8, 12, 16, 20, 24 and 28) body weight, paw volume using a plethysmometer (UGO Basile, Italy), joint diameter using a digital vernier caliper (Mitutoyo digimatic caliper, Japan), thermal hyperalgesia using tail flick analgesy meter (UGO Basile, Italy) and mechanical hyperalgesia was evaluated by Von Frey electronic meter (IITC, life science USA). On 28th day, the animals were sacrificed to study the histological analysis of synovial joint.

(vi) Radiological analysis[19]

At the end of the experiments the hind legs were excised and radiographed on Fuji AGFA film, using a CR-30-X system. Radiographs of each rat were examined for soft tissue swelling, bone matrix resorption, periosteal new bone formation and bone erosion were observed.

(vii) Histological analysis[20,21]

After the X-ray, rats were sacrificed and hind paws were fixed in 10% buffered formalin. The paws were decalcified in 5% formic acid, processed for paraffin embedding, sectioned at 5µm thickness, and subsequently stained with haematoxylin-eosin for examination under a light microscope with 10x magnifications. Sections were observed for the presence of hyperplasia of the synovium, pannus formation and destruction of the joint space.

(viii) Statistical analysis

The data were analyzed by one way ANOVA followed by Dunnett's test, two way ANOVA followed by Bonferroni's post hoc test. All statistical analyses were performed using Graph Pad Prism software (San Diego, CA).

Data was considered statistical significant at P<0.05.

RESULTS

Acute oral toxicity

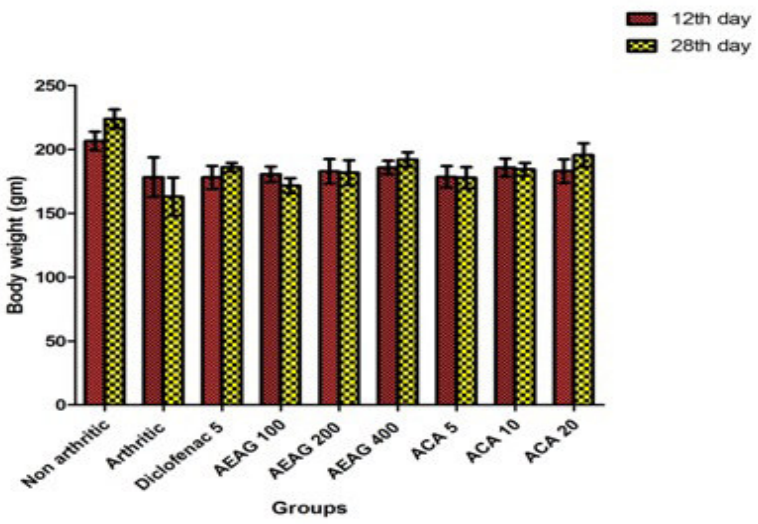
ACA 20 mg/kg and AEAG 2000 mg/kg p.o. did not produce any behavioral abnormalities and mortality. So the doses selected for ACA were 5, 10 and 20 mg/kg and for AEAG were 100, 200 and 400 mg/kg.

(i) Ant-arthritic activity

1. Effects of ACA and AEAG on body weight Body weight of CFA induced arthritic control (group II) rats was less significantly reduced compared to nonarthritic rats (group I) on 28th day. The body weight reduction in ACA (20 mg) and AEAG (400 mg) treated rats were non-significant compared that of arthritic rats (Graph 1).

Graph 1

Effect of oral administration of ACA and AEAG on body weight of arthritic rats.



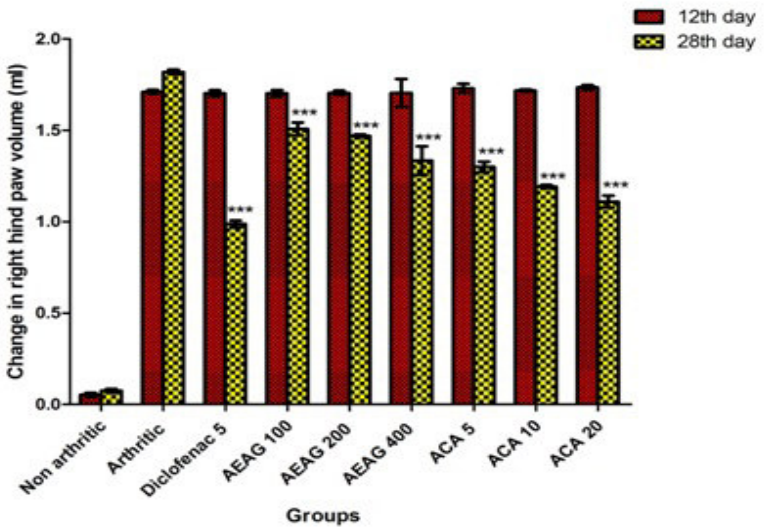
Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001

2. Effects of ACA and AEAG on right hind paw edema

One day after the CFA injection, primary arthritis of the right hind paw was induced, and inflammation was maintained up to 28 days (Graph 2). Oral administration of ACA (5, 10 and 20 mg/kg), AEAG (100, 200 and 400 mg/kg) and Diclofenac (5 mg/kg) from 12th day of arthritis induction significantly (P<0.001) suppressed the increased paw edema up to 28th day.

Graph 2

Effect of oral administration of ACA and AEAG on right hind paw edema in arthritic rats.



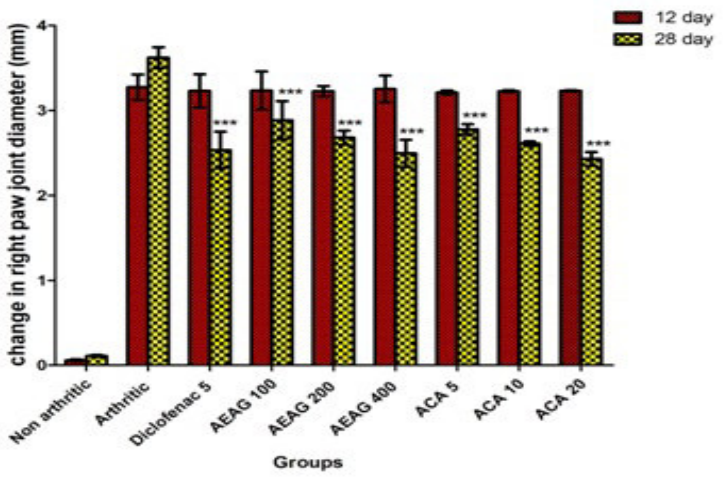
Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

3. Effects of ACA and AEAG on right hind limb joint diameter

The dose-dependent effects of ACA and AEAG are presented in (Graph 3). Administration of CFA in the right hind paw resulted in significant ($P<0.001$) increase in the joint diameter of hind paw compared to that of nonarthritic (control) rats. Diclofenac (5 mg/kg), ACA (5, 10 and 20 mg/kg) and AEAG (100, 200 and 400 mg/kg) showed significant ($P<0.001$) reduction of the right hind limb joint diameter compared with that of arthritic rat on 28th day.

Graph 3

Effect of oral administration of ACA and AEAG on right hind paw joint diameter in arthritic rats.



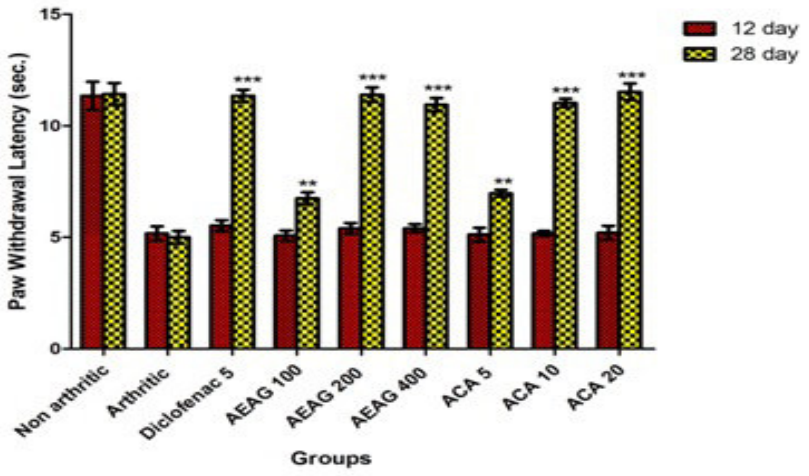
Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

4. Effects of ACA and AEAG on right hind paw thermal hyperalgesia

The paw withdrawal latency (PWL) of the right hind paw of arthritic rats was decreased compared to non arthritic rats. Treatment with diclofenac (5 mg/kg), ACA (10 and 20 mg/kg) and AEAG (200 and 400 mg/kg) showed significant (P<0.001) increase in PWL on 28th day, while the lower doses of AEAG and ACA showed less significant (P<0.01) reduction in the PWL. (Graph 4)

Graph 4

Effect of oral administration of ACA and AEAG on right hind paw withdrawal latency in arthritic rats.



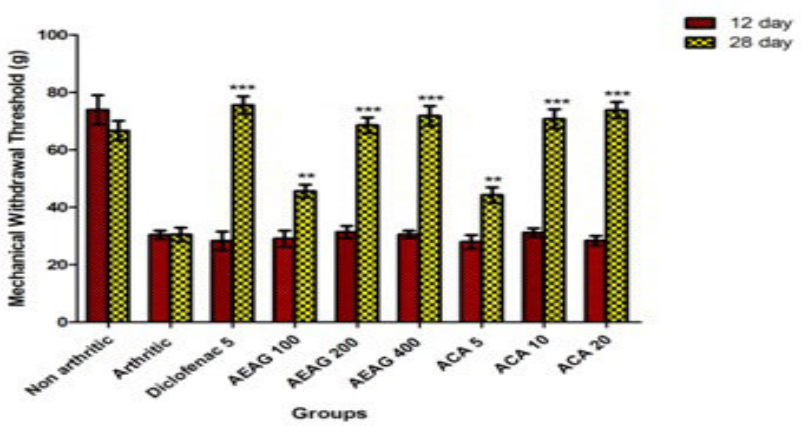
Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

5. Effects of ACA and AEAG on right hind paw mechanical hyperalgesia

The mechanical withdrawal threshold (MWT) of the right hind paw was decreased in arthritic rat compared to the basal level 1 after the CFA injection (graph 5). The oral treatment of diclofenac (5 mg/kg), ACA (10 and 20 mg/kg) and AEAG (200 and 400 mg/kg) significantly (P<0.001) suppressed the MWT on 28th day, while the lower doses of ACA and AEAG showed lesser suppression of the MWT 28th day.

Graph 5

Effect of oral administration of ACA and AEAG on right hind paw mechanical withdrawal threshold in arthritic rats.



Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

6. Histopathology of Synovial joint

Histopathology of synovial joint of normal rats showed intact morphology of synovium. No inflammation and influx of inflammatory cells were observed. CFA treated rats showed cartilage destruction, influx of inflammatory cells, pannus formation, fibrin deposition, synovitis and chronic inflammation. Diclofenac treated rats showed significant protection against cartilage destruction, vascular proliferation and synovial space thickening, low influx of inflammatory cells and no pannus formation. ACA (5 mg/kg) treated rats showed moderate cartilage destruction and synovial space thickening, influx of few inflammatory cells. ACA (10 mg/kg) treated rats showed significant lesser cartilage destruction, synovial space thickening, vascular proliferation, low influx of inflammatory cells and no pannus formation. ACA (20 mg/kg) treated rats showed significant protection against cartilage destruction, vascular proliferation and synovial space thickening, low influx of inflammatory cells and no pannus formation, (Figure 1). AEAG (400 mg/kg) treated rats showed significant lesser cartilage destruction, synovial space thickening, vascular proliferation, low influx of inflammatory cells and no pannus formation. AEAG (200 mg/kg) treated rats showed moderate cartilage destruction and synovial space thickening and influx of few inflammatory cells. AEAG (100 mg/kg) treated rats showed minimal inflammation, influx of few inflammatory cells in synovium with evidence of disturbed synovial lining or pannus formation.

Histopathology of synovial joint

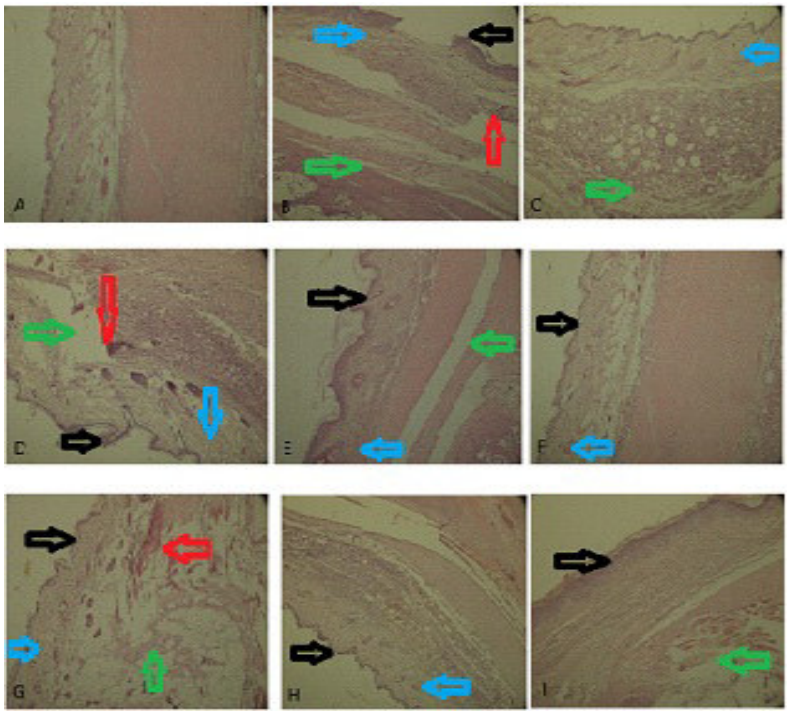


Figure. 1

A) Normal non-arthritic (B) Arthritic control, (C) Diclofenac 5 mg/kg treated, (D) AEAG 100 mg/kg treated, (E) AEAG 200 mg/kg treated, (F) AEAG 400 mg/kg treated. (G) ACA 5 mg/kg (H) ACA 10 mg/kg (I) ACA 20 mg/kg. (Black arrow – synovial lining, Blue arrow – influx of inflammatory cells, Red arrow – pannus formation, Green arrow – cartilage destruction)

7. Radiological analysis

Adjuvant treated rats developed definite joint space narrowing of the intertarsal joints, diffuse soft tissue swelling that included the digits, marked periosteal thickening, cystic enlargement of bone and extensive erosions produced narrowing of all joint spaces. Despite a similar clinical course of

arthritis, CFA control rats suffered from more pronounced bone destruction than AEAG and ACA treated groups, (Figure 2).

Radiological analysis of synovial joint

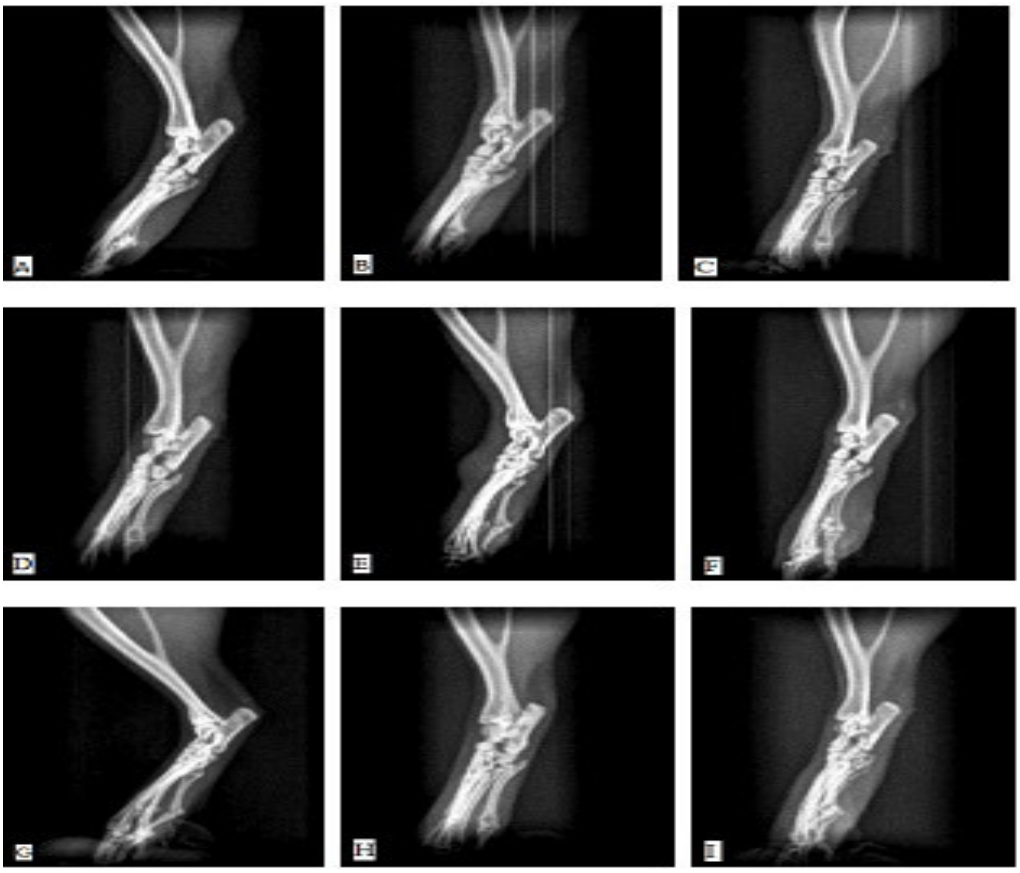


Figure. 2
(A) Non-arthritic (B) Arthritic, (C) Diclofenac (5 mg/kg), (D) AEAG (100 mg/kg), (E) AEAG (200 mg/kg), (F) AEAG (400 mg/kg), (G) ACA (5 mg/kg), (H) ACA (10 mg/kg), (I) ACA (20 mg/kg).

DISCUSSION

Complete Freund's adjuvant induced arthritis is one of the most widely used models for chronic arthritis[22]. Freund's adjuvant (a mixture of heat killed *Mycobacterium tuberculosis* with liquid paraffin) produced inflamed lesions in areas of the body remote from the injection site after a delay of 10 to 15 days[23]. In adjuvant-induced arthritis model rats developed a chronic swelling in multiple joints with influence of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling, it is commonly used for preclinical studies of NSAIDs and anti-rheumatic drugs and this model is most suitable as like human arthritis[24]. Tissue damage following CFA injection is a complex phenomonen, incorporating many different mediators and pathways to produce inflammatory hyperalgesia. Substances known to be involved in this process are products of arachadonic acid metabolism, histamine, 5-HT, bradykinin, cytokines, nitric acid (NO). In rats, CFA, when injected intradermally into the plantar surface of a paw, produces a characteristic inflammation and associated hyperalgesia, which can be used to quantify the anti-inflammatory or anti-hyperalgesic actions of drugs[25]. Inflammatory mediators, like bradykinin, which are released from injured tissue, sensitize and directly stimulate nociceptors, and stimulate tumour necrosis factor-alpha (TNF-α) release. The

TNF-α in turn, stimulates the release of interleukin-1beta (IL-1β) and interleukin-6 (IL-6), promoting the induction of cycloxygenase enzymes, which convert arachidonic acid to prostaglandins. Tumour necrosis factor-alpha also stimulates the release of cytokine-induced neutrophil chemoattractant (CINC-1) in rats or interleukin-8 (IL-8) in humans. Cytokines, like IL-1β, TNF-α and IL-6, contribute to the development of hyperalgesia by sensitizing peripheral nociceptors, decreasing the peripheral nociceptor threshold[26]. As per the results of our study ACA and AEAG exhibited a significant anti-arthritic activity by inhibition of paw volume and reduced joint diameter in arthritic treated rats. ACA and AEAG also show significant anti- hyperalgesic activity. The actual mechanism may be due to the suppression of inflammatory mediators like IL-1β, TNF-α and IL-6 released due to induction of Freund's adjuvant in arthritic rats. 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 development stages (final stages) of arthritis[27]. The standard drug diclofenac prevented the bony destruction and also there was no swelling of the joint. AEAG (400 mg/kg) and ACA (20 mg/kg) treatment for 28 days, prevented bony destruction as there was less soft tissue swelling and narrowing of joint spaces than that observed on 14th day. Petchi and Vijaya also supported FCA induced arthritis model for evaluation of antiartheritic activity[28].

CONCLUSION

The results of the present study demonstrate that ACA and acetone extract of *A. galanga* rhizomes (AEAG) possess anti-arthritic activities in animals. The possible mechanisms may be due to inhibition of release of mediators of inflammation and antioxidant activity of ACA. Further studies are required to find out the actual mechanism of action.

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