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Antinociceptive, anti inflammatory and antiarthritic activity of ethanol root extract and fraction of *Aganosma dichotoma* (Roth) K. Schum

Deepali Pandey[1], Apurva Joshi[1], Shardendu Mishra[2], Krisnamurthy Sairam[1] & S Hemalatha[1]*

[1]Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi-221 005, Uttar Pradesh, India
[2]Department of Pharmacology, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221 005, Uttar Pradesh, India

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Aganosma dichotoma (Roth) K. Schum, locally called Malati, has been traditionally used in the treatment of pain and inflammation in Ayurvedic system of medicine. In the present study, We investigated its antiarthritic potential. Qualitative and quantitative study through HPTLC was done in ethanolic root extract (EAD), petroleum ether fraction (PF) and chloroform fraction (CF) using quercetin, kaempferol, ursolic acid and lupeol as standard. EAD (100, 200 and 400 mg/kg, p.o.), PF and CF (100 and 200 mg/kg, p.o.) were evaluated for analgesic, anti-inflammatory and antiarthritic activities by Freund's complete adjuvant induced arthritis model (FCA). Arthritis was assessed on the basis of 'change in paw' volume and arthritis index. Hematological parameters, spleen and thymus index, cytokine level in serum, radiological and histological parameters were also evaluated. EAD possess a higher amount of ursolic acid (2.23%) and lupeol (5.81%). EAD 400 mg/kg, p.o. showed potent inhibition in paw volume and TNF-α (33.19, 57.32%) as compared to curcumin 100 mg/kg, p.o. (31.12, 44.09%), respectively. EAD, PF and CF possessed significant analgesic, anti-inflammatory and antiarthritic activities. EAD and PF at higher dose decreased the spleen index.

Keywords: Antiarthritic, Ayurvedic, Freunds adjuvant induced arthritis, Inflammation, Madhumaalati, Pain, Rheumatoid arthritis

Rheumatoid arthritis (RA) is a common autoimmune disorder characterized by chronic inflammation, synovial membrane inflammation and destruction of joints due to progressive attrition of articular cartilage in synovial joint through generations and infiltration of auto antibodies. In Indian subcontinent, around 0.4-0.6% of the population is affected by RA, whereas the worldwide prevalence is 1% of the adult population. Its occurrence is three times more in women as compared to men[1]. Pathophysiology of RA is associated with the T cells, B cells and the orchestrated interaction of pro-inflammatory cytokines. TNF-α, IL-1, IL-6 and IL-17 are the cytokines involved where they may be either directly involved in the process or play a major role in cell migration and inflammation in RA[2]. The current approach for RA treatment is mainly associated with the reduction of pain and inflammation using non-steroidal anti-inflammatory drugs (NSAIDs) with disease-modifying anti rheumatic drugs (DMARDs) supplements, steroid hormone and biologicals (TNF-α antibody and the decoy TNF-α receptor, etc.) which suppress the immunological processes involved in the progression of RA. Hence the use of NSAIDs and DMARDs in combination is a more efficient approach towards the treatment of RA. However, besides being expensive, they also inflict severe well known adverse effects such as gastrointestinal ulcergenicity, teratogenesis, cardiovascular complications and renal morbidity[3]. Therefore, contemporary research focus is shifted towards the development of novel, efficient and herbal anti arthritic agent with higher efficacy and minimum side effects.

Aganosma dichotoma (Roth) K. Schum (Apocynaceae), is a large climber widely distributed across India, China, Philippines and Indonesia. In India, it extends throughout Assam, Bihar, West Bengal, Orissa, Andhra Pradesh and Tamil Nadu. *A. dichotoma* has been traditionally used in various ailments, including emesis, anthelmintic, bronchitis, leprosy, skin disease, ulcers, inflammation and disease of mouth while flowers are used in disease of eye and leaves are used in biliousness[4,5]. It is also used as antiseptic, anodyne and also used as an ingredient in massage oils for paraplegia, neuralgia, sciatica[6]. Our previous investigation reveals quality control standardization, anti urolithic and anti ulcer activities of *A. dichotoma*[7-9]. The present study aims to scientifically validate the traditional claims of *A. dichotoma* as analgesic and

*Correspondence:
E-mail: shemalatha.phe@itbhu.ac.in

anti inflammatory as well as to investigate its antiarthritis potential.

Materials and Methods

Plant material

The roots of *A. dichotoma* were collected from Tumbura Kona Kshetram at Seshachalam hills and Tirumala hills, Chittor District, Andhra Pradesh in the month of April 2013 and were authenticated by Dr. K. Madhava Chetty, Taxonomist, SV University, Tirupati. A voucher specimen (COG/AD/17) has been kept in Department of Pharmaceutics, IIT-BHU, Varanasi for further reference.

Preparation of extract and fractions

After shade drying, the dried roots (1 kg) were coarsely grounded into homogenous powder using a mechanical grinder, passed through a 60 mesh sieve and exhaustively extracted with 95% ethanol (3 L) in a soxhlet apparatus for 72 h. The EAD was filtered and concentrated with rota evaporator (IKA). The dried EAD (100 g) was made hydroalcoholic (7:3) and then subjected to successive fractionation using solvents of increasing polarity such as petroleum ether (PF), chloroform (CF) and ethyl acetate (EAF). The fractions were concentrated under reduced pressure in a rotary evaporator and were then kept in desiccator until use. The yield of the fractions reported were PF, 9.49% (w/w); CF, 3.05% (w/w); and EAF, 1.17% (w/w). After preliminary screening for analgesic and anti-inflammatory activity, only petroleum ether and chloroform fractions (PF and CF) were selected for the study.

HPTLC fingerprinting analysis and quantification of EAD, PF and CF

Extract, PF and CF were dissolved in chromatography grade methanol (1 mL) and solvent system was optimized. A solvent system consisting of Toluene:Ethyl acetate:Formic acid (7:3:0.5 v/v/v) was used to resolve and quantify the characterized compounds[8,10]. Sample application was done and the plate was run in the above given solvent system, then chromatogram was developed at 350 nm after that plate was derivatized by anisaldehyde-sulfuric acid reagent followed by development of chromatogram which was scanned by densitometer and R_f value and fingerprint data were recorded by WINCATS software. Presence of kaempferol, quercetin, ursolic acid, beta sitosterol and lupeol in the extract and fractions was confirmed with the help of R_f value and spectral comparison using standards and were quantified in EAD, PF and CF.

Animals

Adult Charles Foster albino rats (150±10 g) of either sex, were used for the study and pharmacological experiments were approved by the Central Animal Ethical Committee, Institute of Medical Sciences, Banaras Hindu University (Approval no.: Dean/2015/ CAEC/983).

Acute toxicity study

Acute oral toxicity studies of EAD, PF and CF were performed and administered orally up to 2000 mg/kg body weight. Animals were closely observed for the initial 4 h after the administrations, and then once daily up to 14 days[11].

Antinociceptive activity

Acetic acid induced writhing test

Rats were divided into 10 groups (6 rats in each group). Group I was control while group II and III animals were treated with standards indomethacin [Sigma aldrich (purity: 99%)] (5 mg/kg, p.o.) and curcumin [Ranbaxy (purity: 95%)] (100 mg/kg, p.o.) respectively. Animals in group IV-VI received EAD 100, 200 and 400 mg/kg, p.o., while group VII and VIII received PF 100 and 200 mg/kg, p.o., and group XI and X animals were treated with CF 100 and 200 mg/kg, p.o. After 45 min of drug administration, each rat was injected with acetic acid i.p. (0.8%, v/v, 10 mL/kg). The number of writhing response was recorded after 15 min of acetic acid administration and percentage inhibition of writhing was calculated[12].

Formalin induced pain

After 30 min of drug administration (grouping was similar to the previous model), pain was induced by injecting 0.05 mL of 2.5% formalin in distilled water in the sub-plantar of the right hind paw. The number of lickings from 0 to 5 min (first phase) and 15-30 min (second phase) were counted[13].

Tail flick test

The tail flick test was conducted according to the method described by D'Amour and Smith (1941)[14], where animal grouping was kept similar to the writhing test but morphine sulfate [Drugs India Pvt. Ltd. Dispur, Guwahati-5 (purity: 96%)] (5 mg/kg, p.o.) was used as standard drug in this model. The rat's tail was placed in the window of the tail flick apparatus (UGO Basile, Italy) and reaction time was

recorded before and at 30, 60 and 90 min in both treated and control groups.

Anti-inflammatory activity

Carrageenan induced rat paw edema

One hour after the drug administration, edema was induced by carrageenan injection (0.1 mL, 1%, w/v in saline) into the sub-plantar tissue of the left hind paw and grouping was similar to writhing test. Paw volume was measured by plethysmometer at 0^{th}, 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 6^{th} h after carrageenan injection[15].

Cotton pellet induced granuloma

According to Swingle and Shideman (1972)[16], granulomatous lesions were induced by surgically implanting two cotton pellets subcutaneously in the dorsal region of the rats; one near each axilla and grouping was same as in writhing test. EAD, PF and CF were administered once daily throughout the experimental period of 7 days. On the 8^{th} day, cotton pellets were removed surgically. Extraneous tissue was removed from pellet and dried at 60ºC for 18 h, until the weight became constant. The mean weight of the cotton pellets of the control group as well as of the treatment groups was calculated[16].

Antiarthritic activity

Freunds adjuvant induced arthritis

Arthritis was induced by single 0.1 mL intra dermal injection of Freund's complete adjuvant (FCA, Sigma Aldrich) in foot pad of the left hind paw of rats[17]. Animals were divided into 11 groups with six no. of animal in each group. Group I, normal control; Gr. II, FCA induced arthritic rats; Gr. III, FCA+ methotrexate [Cadila Pharmaceutical (purity: 98%)] (3 mg/kg, p.o.); Gr. IV, FCA+curcumin (100 mg/kg, p.o.); Gr. V-VII, FCA+EAD (100, 200, 400 mg/kg, p.o); Gr. VIII and IX, FCA+PF (100 and 200 mg/kg, p.o); and Gr. X and XI, FCA+CF (100 and 200 mg/kg, p.o.). Treatment of rats was started with EAD, PF and CF from day 2 and continued up to 28 days.

Evaluation of body weight, paw volume and arthritic score

The change in body weight and left hind paw volumes of all animals were estimated just before the administration of FCA injection and thereafter at every 7^{th} day time intervals up to day 28. The morphological aspects of arthritis like redness, swelling, erythema and use of joint was monitored by set visual criteria as follows: normal paw = 0, mild swelling and erythema = 1, low to moderate swelling and erythema = 2, severe swelling and erythema with limited joint use = 3, gross deformity and inability to use the limb = 4 on respective days[18].

Hematological parameters

The hematological parameters, for instance haemoglobin, RBCs, WBCs and ESR were determined by standard methods[19].

Evaluation of thymus and spleen index

The thymus and spleen index was articulated as the ratio (mg/*g*) of thymus and spleen wet weight versus body weight, respectively[20].

Antioxidant parameters

The ankle tissue of rats was isolated and washed in ice cold saline. Tissue homogenates were prepared with 0.1 M Tris HCl buffer (pH 7.4) and centrifugation was done initially at 800 ×g for 10 min and later at 12000 ×g for 15 min. The supernatant obtained was used to estimate SOD, LPO, CAT and GSH[21].

Estimation of cytokine level

Rat blood was collected and allowed to cool centrifuge with 3000 rpm at 4ºCfor 15 min. Serum was recovered and frozen at −20ºC until assayed. Concentration of TNF-α, IL-1β and IL-6 were assessed in serum by ELISA (Koma Biotech) as per manufacturer's protocol.

Radiological analysis of ankle joints

The left hind paw of anesthetized rats was radiographed on the 28^{th} day by digital X-ray (Brivo XR 115 unit, Wipro GE healthcare). Radiographic analysis of hind paws was performed at 200 kHz peak, 100 mA and the exposure time was 5 s.

Histopathology of ankle joints

On 28^{th} day, ankle joints were alienated from the hind paw and immersed in 10% buffered formalin for 24 h followed by decalcification in 5% formic acid, processed for paraffin embedding sectioned at 3 µ thickness. The sections were stained with haematoxylin eosin and evaluated for the presence of inflammatory cells, hyperplasia of synovium, pannus formation and destruction of joint space[22].

Statistical analysis

All data are expressed as mean ± SEM with n=6 per group. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett's post test as well as two-way ANOVA followed by Bonferroni post test.

Results

HPTLC fingerprinting analysis and quantification of EAD, PF and CF

HPTLC fingerprinting of EAD is depicted in Fig. 1A which shows 4 peaks and 7 peaks in Fig. 1B. Presence of kaempferol (R_f 0.56) and quercetin (R_f 0.59) was observed at 350 nm and confirmed by R_f value and spectral comparison using marker compounds. Fig. 1B, observed after derivatization of plate, indicates the presence of ursolic acid (R_f 0.34), beta sitosterol (R_f 0.63) and lupeol (R_f 0.78). Fig. 1C and 1D represent the fingerprinting data of CF while 1E represent the fingerprinting of PF. Triterpenoids i.e. ursolic acid, beta sitosterol and lupeol were present in PF while kaempferol and quercetin were absent. Quantitative study of kaempferol, quercetin, ursolic acid, beta sitosterol and lupeol were carried out by HPTLC and found to be EAD (0.523, 2.07, 1.26, 2.23 and 5.81% w/w), CF (1.06, 3.86, 1.59, 1.84 and 2.61% w/w) PF (1.63, 1.06 and 4.93% w/w), respectively.

Acute toxicity study

Oral acute toxicity study of EAD, PF and CF did not show any signs of toxicity and mortality up to 2 g/kg during the desired observation period.

Antinociceptive activity

The inhibition rate of the number of writhes for the EAD (100, 200 and 400 mg/kg, p.o.) were (21.90, 57.64, 82.87%) while for PF (100 and 200 mg/kg, p.o.) were (18.37, 54.18%) and for CF (100 and 200 mg/kg, p.o.) were (16.82, 40.93%). Percentage inhibition of writhes exhibited by EAD at the dose of 400 mg/kg, p.o. (82.87%) was comparable to the standard drug indomethacin 5 mg/kg, p.o. (80.69%), whereas higher than curcumin 100 mg/kg p.o. (60.37%) treated animals. (Fig. 2A)

Table 1 shows that EAD at 400 mg/kg, p.o., PF (100 and 200 mg/kg, p.o.) and CF (100 and

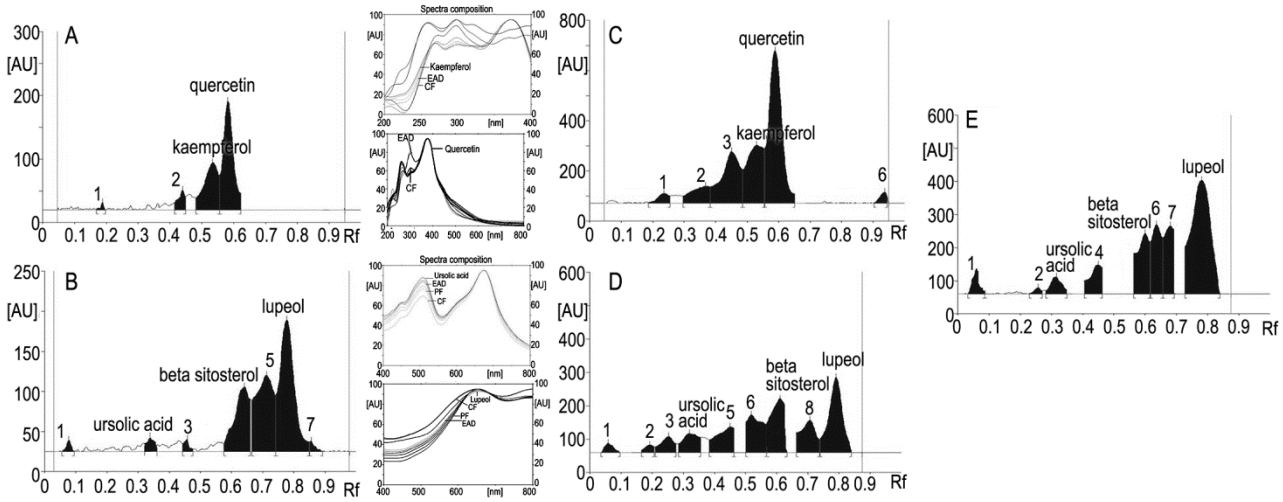

Fig.1 — HPTLC fingerprinting of ethanolic root extract (EAD), petroleum ether fraction (PF) and chloroform fraction (CF)

Table 1 — Effect of ethanolic root extract (EAD), petroleum ether fraction (PF) and chloroform fraction (CF) on formalin induced pain

Group	First Phase	% Inhibition	Second Phase	% Inhibition
Control (0.5% CMC)	77.00±2.36	-	57.50±1.88	-
Indomethacin (5 mg/kg)	22.33±1.28^{c}	71.00	10.50±0.99^{c}	81.73
Curcumin (100 mg/kg)	26.16±1.62^{c}	66.02	18.33±1.35^{c}	68.12
EAD (100 mg/kg)	72.33±1.33	6.06	49.61±1.22^{b}	13.72
EAD (200 mg/kg)	69.78±1.70^{a}	9.37	41.52±1.81^{c}	27.79
EAD (400 mg/kg)	22.16±1.66^{c}	71.22	9.43±1.11^{c}	83.60
PF (100 mg/kg)	61.80±1.86^{c}	19.74	45.34±1.89^{c}	21.14
PF (200 mg/kg)	56.50±1.14^{c}	26.62	28.61±1.67^{c}	50.24
CF (100 mg/kg)	68.23±1.71^{b}	11.68	46.82±1.54^{c}	18.57
CF (200 mg/kg)	54.82±1.25^{c}	28.80	37.71±1.91^{c}	34.41

[All statistical data are expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test. ^{a,b,c} Significance as compared to control P <0.05, <0.01 and <0.001, respectively]

200 mg/kg, p.o.) produced a significant dose dependent inhibition of neurogenic phase (0-5 min), while EAD, PF and CF at all dose levels showed significant action on inflammatory phase (15-30 min) of formalin induced licking.

Fig. 2B represents analgesic activity of EAD, PF and morphine. EAD (100, 200 and 400 mg/kg, p.o.) and PF (100, 200 mg/kg, p.o.) showed significant effect after 60 min and increased latency in flicking tail. The observed effect was found to be more pronounced (P <0.001) in rats treated with EAD at (100, 200, 400 mg/kg, p.o.), PF 100 and 200 mg/kg, p.o. (P <0.05, P <0.01) which was quite comparable with standard morphine while CF at any dose did not show significant effect on latency in flicking.

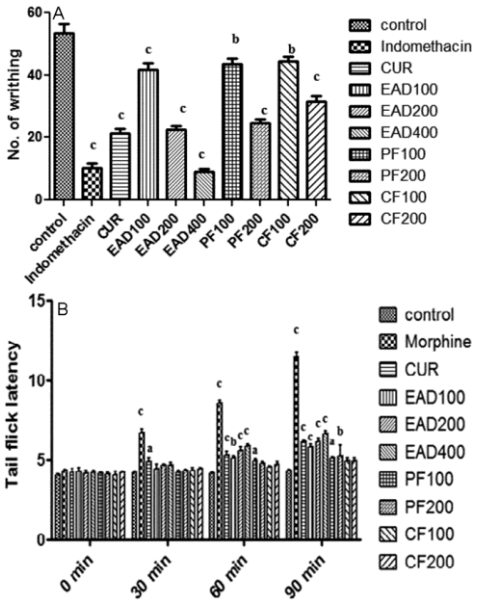

Fig. 2 — Effect of extract and fractions on (A) acetic acid induced writhing test. [All statistical data are expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test. [b]P <0.01 statistically significant as compared to control. [c]P <0.001 statistically significant as compared to control]; and (B) tail flick method. [All statistical data was expressed in mean ± SEM and determined by Two way ANOVA followed by Bonferroni post test. [a] P <0.05 statistically significant as compare to control; [b]P <0.01 statistically significant as compared to control; [c]P <0.001 statistically significant as compare to control]

Anti-inflammatory activity

Table 2 showed that EAD at 200 and 400 mg/kg, p.o. doses significantly (P <0.001) inhibited the carrageenan-induced paw edema from 3 to 5 h, while EAD 100 mg/kg, p.o. produced significant action only at 5 h. PF and CF (100 and 200 mg/kg) significantly (P <0.001) inhibit the paw volume from 3-5 h. The maximum inhibitory effect at 3h and 5h of the extract was recorded at the 400 mg/kg, p.o. dose (35.82, 46.86%) as compared to indomethacin (38.05, 45.38%) and curcumin (21.64, 38.37%), respectively.

Table 3 shows that the EAD (200 and 400 mg/kg, p.o.), PF and CF (200 mg/kg, p.o.) exhibited significant

Table 3 — Effect of EAD, PF and CF on cotton pellet granuloma in rats

Groups	Dose (mg/kg, p.o.)	Granuloma dry weight (mg)	% Inhibition
Control	0.5%CMC	89.26±1.78	-
Indomethacin	5	52.94±1.94[c]	40.69
Curcumin	100	58.79±1.22[c]	34.13
EAD	100	81.53±2.78	8.66
EAD	200	68.45±2.56[c]	23.31
EAD	400	51.33±1.56[c]	42.49
PF	100	79.47±2.54[a]	10.96
PF	200	68.91±1.22[c]	22.79
CF	100	81.34±2.82	8.87
CF	200	74.56±1.66[c]	16.46

[All statistical data are expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test. [a] P <0.05 statistically significant as compared to control; [b]P <0.01 statistically significant as compared to control; and [c]P <0.001 statistically significant as compared to control]

Table 2 — Effect of different doses of ethanolic root extract (EAD), petroleum ether fraction (PF) and chloroform fraction (CF) on carrageenan induced paw edema

Groups	Dose (mg/kg, p.o.)	Left hind paw volume (ml)					
		0 h	1 h	2 h	3 h	4 h	5 h
Control	0.5%CMC	1.42±0.08	1.84±0.08	2.23±0.14	2.68±0.10	2.65±0.10	2.71±0.04
Indomethacin	5	1.39±0.04	1.46±0.04[c]	1.61±0.04[c]	1.66±0.02[c]	1.65±0.03[c]	1.48±0.04[c]
Curcumin	100	1.44±0.04	1.61±0.03[a]	1.93±0.04[b]	2.10±0.05[c]	1.88±0.03[c]	1.67±0.03[c]
EAD	100	1.40±0.03	1.80±0.01	2.15±0.02	2.47±0.10	2.43±0.14	2.21±0.15[c]
EAD	200	1.39±0.03	1.75±0.06	2.06±0.05	2.28±0.10[c]	2.11±0.07[c]	1.86±0.05[c]
EAD	400	1.38±0.05	1.54±0.05[b]	1.68±0.04[c]	1.72±0.05[c]	1.69±0.02[c]	1.44±0.03[c]
PF	100	1.41±0.01	1.51±0.06[c]	1.81±0.02[c]	2.06±0.03[c]	1.96±0.02[c]	1.95±0.05[c]
PF	200	1.41±0.06	1.61±0.02[a]	1.99±0.04[a]	1.96±0.02[c]	1.91±0.03[c]	1.71±0.03[c]
CF	100	1.45±0.04	1.78±0.03	2.48±0.06[a]	2.24±0.05[c]	2.13±0.04[c]	1.98±0.04[c]
CF	200	1.39±0.04	1.73±0.06	1.84±0.08[c]	2.19±0.02[c]	1.99±0.06[c]	1.87±0.02[c]

[All statistical data are expressed in mean ± SEM and determined by Two way ANOVA followed by Bonferroni post test. [a,b,c] Significance as compared to control P <0.05, <0.01 and <0.001, respectively]

(P <0.001) and dose dependent inhibition of dry weight of the cotton pellet granuloma. EAD 400 mg/kg, p.o. dose was displayed higher % inhibition in granuloma formation (42.49%) than the standard drugs indomethacin (40.69%) and curcumin (34.13%).

Antiarthritic activity

Rats of the control group showed a marked weight loss in the second week after Freund's adjuvant injection, followed by normal weight gain in subsequent weeks. However, methotrexate (MTX), curcumin, EAD, PF and CF treated groups did not show significant weight loss (Fig. 3A). Administration of EAD 100 and 200 mg/kg, p.o. significantly decreased the paw volume from 14 to 28 days as compared to control group while PF and CF at all dose levels inhibit the paw volume from 21 to 28 days as shown in Fig. 3B. MTX (34.43%), curcumin (31.12%) and EAD 400 mg/kg, p.o. (33.19%) shows maximum % inhibition of paw volume on 28 days respectively. The reduction of clinical arthritis score was observed in the group treated with EAD, PF and CF at all doses and demonstrated significant effect throughout the treatment period from 21stday. MTX and curcumin showed potent efficacy from day 14 to 28. (Fig. 3C)

Table 4 represents the changes in hematological parameters in FCA induced arthritic rats. As shown in Fig. 4, only curcumin, EAD (200 and 400 mg/kg, p.o.) and PF 200 mg/kg, p.o. treated rats exhibited a significant (P <0.01) decrease in spleen index as compared to control group whereas, curcumin, EAD, PF and CF had no significant effect on thymus index. MTX (3 mg/kg, p.o.) treated rat decreased both thymus and spleen index.

Different biochemical parameters were evaluated in the FCA induced model (Table 5). The results elicited a significant (P <0.05) reduction in lipid peroxidation (LPO) while an increase in SOD, GSH and CAT level in serum.

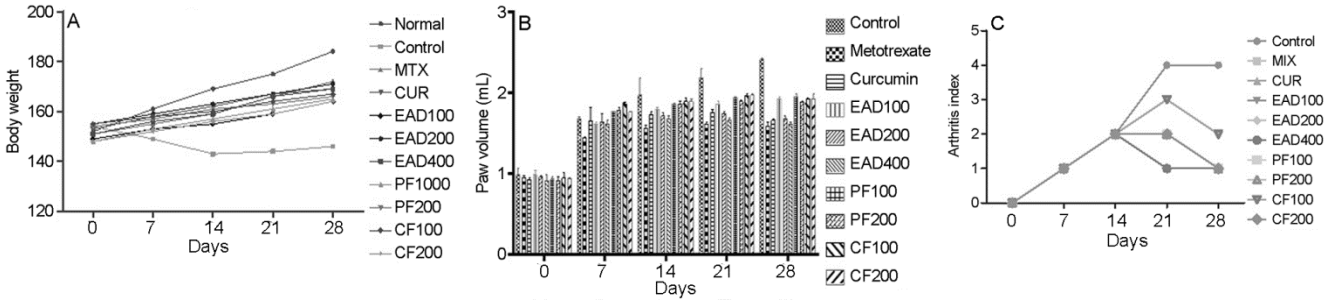

Fig. 3 — Effect of EAD, PF and CF on change in (A) body weight, (B) paw volume; (C) and arthritic index in FCA induced arthritis. [(A) All statistical data was expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test. Only control group shows significant weight loss (P <0.001) on 14 days as compare to normal rats. MTX, curcumin (CUR), EAD, PF and CF treated rat shows nonsignificant action on body weight as compare to normal rats; (B) Values are expressed as mean ± SEM for six animals and analysed by two-way ANOVA followed by Bonferroni post test. MTX, curcumin (CUR), EAD, PF and CF at all dose level shows significant change in paw swelling (P <0.001) on 28 days. While only MTX shows (P <0.05) significant action on 7th day; and (C) Values are expressed as mean ± SEM (n=6) and analysed by two-way ANOVA followed by Bonferroni post test. MTX, curcumin (CUR), EAD, PF and CF at all dose level shows significant action (P <0.001) on arthritis index as compared to control group from 21st to 28th days]

Table 4 — Effect of EAD, PF and CF on Hematological parameters

Groups	Dose (mg/kg, p.o.)	RBC (millions/mm^3)	WBC (thousands/mm^3)	Hb (g/dl)	ESR (mm/h)
Control	0.5% CMC	4.63±0.38	25.63±1.28	8.11±1.72	9.44±1.34
MTX	3	7.57±0.21^c	14.21±0.89^c	13.38±0.52^b	4.87±0.48^b
Curcumin	100	7.14±0.38^b	16.16±0.63^c	13.61±0.34^c	4.23±0.41^c
EAD	100	5.82±0.41	21.28±1.78^a	11.26±1.12	6.66±0.93
EAD	200	6.69±0.27^a	17.63±0.37^c	13.17±0.37^b	5.12±0.61^b
EAD	400	7.13±0.46^b	15.11±0.38^c	14.61±0.28^c	4.93±0.34^b
PF	100	6.92±0.29^b	17.67±0.93^c	12.79±1.23^b	5.83±0.78^a
PF	200	7.22±0.34^b	16.48±0.73^c	13.46±0.78^b	5.11±0.52^b
CF	100	5.72±0.89	20.61±1.26^b	12.24±0.97^a	6.98±1.06
CF	200	6.39±0.61	19.72±1.12^b	12.98±0.87^b	5.87±0.88^a

[All statistical data are expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test. ^{a} P <0.05 statistically significant as compared to control; ^{b} P <0.01 statistically significant as compared to control; and ^{c} P <0.001 statistically significant as compared to control]

Table 5 — *Invivo* antioxidant effect of EAD, PF and CF

Groups	Dose (mg/kg, p.o.)	LPO(MDA, nmol/g tissue)	SOD (units/g tissue)	CAT (units/g tissue)	GSH (µg GSH/g tissue)
Normal		74.46±1.67	62.32±0.97	38.43±1.46	234.78±3.11
Control	0.5%CMC	97.51±1.22^{a}	20.37±0.48^{a}	11.78±1.92^{a}	104.61±4.23^{a}
MTX	3	38.89±1.46^{ab}	57.36±1.78^{b}	36.41±0.97^{b}	238.31±4.36^{b}
Curcumin	100	45.61±1.21^{ab}	48.39±1.27^{abc}	31.78±1.67^{b}	226.71±3.56^{b}
EAD	100	86.93±1.53^{abcd}	21.40±0.38^{acd}	14.56±1.98^{acd}	134.67±4.78^{acd}
EAD	200	67.48±1.17^{bcde}	37.53±0.47^{abcde}	25.61±1.23^{abce}	197.61±3.55^{abce}
EAD	400	43.73±1.60^{abef}	58.22±0.59^{bdef}	34.51±1.78^{bef}	218.09±2.67^{be}
PF	100	72.34±1.76^{bcdeg}	28.15±1.54^{abcdefg}	21.76±1.41^{abcdf}	198.46±3.18^{be}
PF	200	54.65±1.34^{abcdefgh}	46.38±1.72^{abcefgh}	28.77±1.41^{abce}	217.56±3.85^{be}
CF	100	61.82±1.89^{abcdeghi}	31.38±0.89^{abcdefgi}	19.38±1.19^{abcdfh}	187.20±3.24^{abce}
CF	200	58.44±1.34^{abcdefg}	39.78±0.75^{abcdeghij}	26.75±1.67^{abce}	215.23±3.81^{be}

[All statistical data are expressed in mean ± SEM and determined by one way ANOVA followed by Tukey's multiple comparison test. [a] P <0.05 statistically significant as compared to normal control; [b] P <0.05 statistically significant as compared to negative control; [c] P <0.05 statistically significant as compared to MTX; [d] P < 0.05 statistically significant as compared to curcumin; [e] P < 0.05 statistically significant as compared to EAD 100; [f] P <0.05 statistically significant as compared to EAD 200; [g] P <0.05 statistically significant as compared to EAD 400; [h] P <0.05 statistically significant as compared to PF 100; [i] P <0.05 statistically significant as compared to PF 200; and [j] P <0.05 statistically significant as compared to CF 100]

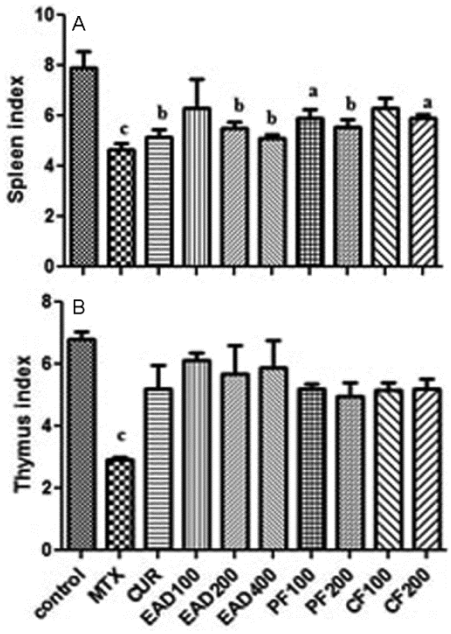

Fig. 4 — Effect of EAD, PF and CF on spleen and thymus index. [Values are expressed as mean ± SEM (n=6) and determined by one way ANOVA followed by Dunnett's post test. [a]P <0.05 statistically significant as compared to control. [b]P <0.01 statistically significant as compared to control; [c]P <0.001 statistically significant as compared to control]

MTX, curcumin, EAD, PF and CF showed a significant effect and decrease the production of TNF-α, IL-1β and IL-6 as compared to control group shown in Table 6. Furthermore, MTX, curcumin and EAD 400 showed potent inhibition (61.14, 44.09 and 57.32%) in TNF-α level, respectively.

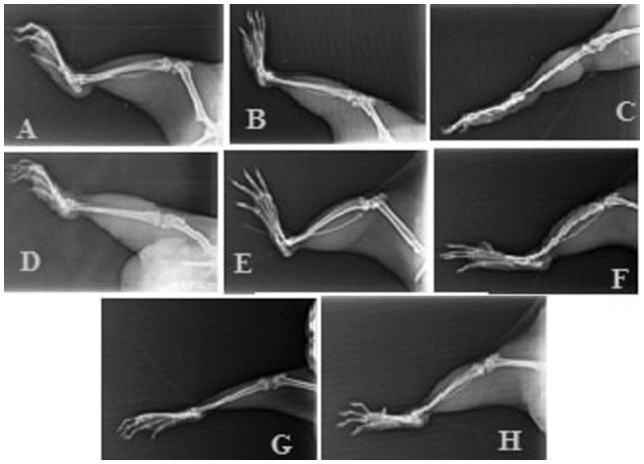

Fig. 5 — Radiological analysis of ankle joint. (A) arthritic control; (B) MTX (3 mg/kg, p.o.); (C) curcumin (100 mg/kg, p.o.); (D-F) EAD (100, 200 and 400 mg/kg, p.o.); (G) PF 200 mg/kg; and (H) CF 200 mg/kg treated rats.

From radiological analysis of ankle joint, it was evident that FCA induced group produced an observable sign of inflammation represented as ankylosis, osteophytes formation, bone erosion and subchondral cyst formation. EAD, PF and CF showed the suppression of inflammation and subsequent arthritic joint development. Rats treated with EAD 400 mg/kg, p.o. dose showed a marked increase in joint space and reduced the bone erosion with less distorted metatarsal joint. MTX and curcumin treated group did not show any visible sign of bone erosion and joint deformation but mild sign of inflamed tissue were observed (Fig. 5).

Table 6 — Effect of extract and fractions on cytokine levels (TNF-α, IL-1β and IL-6) in serum

Groups	Dose (mg/kg, p.o.)	IL-1β (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)
Control	0.5% CMC	128.51±3.26	294.51±39.27	68.22±4.12
MTX	3	56.17±2.54[c]	78.94±44.61[c]	26.51±1.79[c]
CUR	100	69.84±5.04[c]	93.44±38.44[b]	38.14±2.05[c]
EAD	100	117.12±7.32	164.88±31.25	66.59±2.14
EAD	200	98.34±5.72[b]	134.51±44.29[a]	42.27±1.56[c]
EAD	400	62.19±3.44[c]	104.78±29.16[b]	29.11±1.38[c]
PF	100	97.45±3.19[b]	143.72±34.55[a]	53.10±0.92[c]
PF	200	77.54±8.12[c]	119.79±28.92[b]	36.33±1.24[c]
CF	100	106.72±7.45[a]	136.53±22.45[a]	58.19±0.78[b]
CF	200	89.41±3.44[c]	124.57±34.08[b]	36.21±2.16[c]

[All statistical data are expressed in mean ± SEM and determined by one way ANOVA followed by Dunnett's post test. [a] P <0.05 statistically significant as compared to control; [b]P <0.01 statistically significant as compared to control; and [c]P <0.001 statistically significant as compared to control]

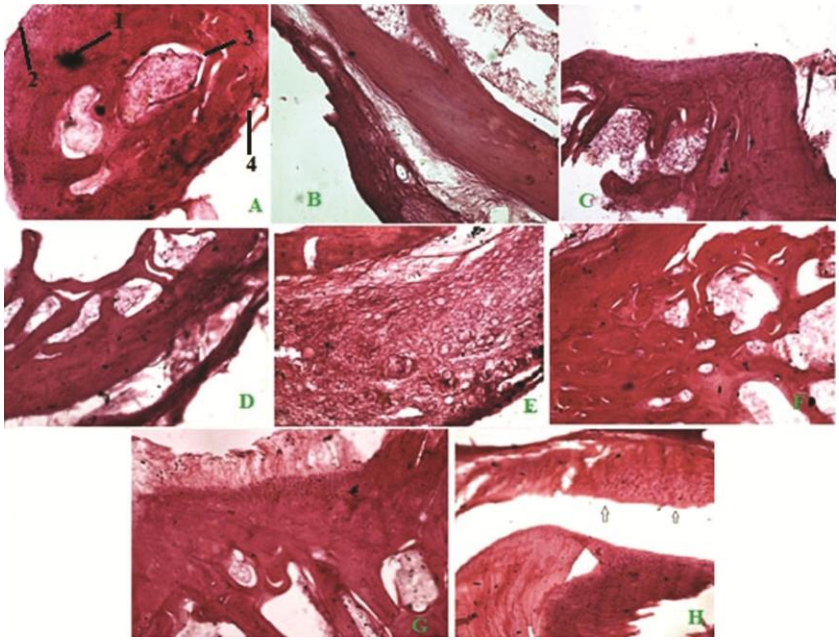

Fig. 6 — Histology of ankle joint of adjuvant induced arthritic rats. (A) arthritic control; (B) MTX treated; (C) curcumin; (D-F) EAD (100, 200 and 400 mg/kg); (G) PF 200 mg/kg; and (H) CF 200 mg/kg treated rats. [(Indication of arrow) 1: Dense mononuclear cell infilteration, 2: Damaged articular cartilage, 3: Pannus formation, 4: Synovial membrane proliferation]

Histopathological evaluation of the ankle joint in an FCA group is shown in Fig. 6 which demonstrated mononuclear cell infiltration, damaged articular cartilage and subchondral bone, pannus formation and synovial membrane proliferation. The rats treated with EAD showed significantly reduced pathological changes in a dose dependent manner as compared to control group. Reduced pannus growth and synovial erosion was also observed in PF 200 mg/kg, p.o. dose treated rats while CF 200 mg/kg, p.o. treated rats showed influx of inflammatory cells with disturbed synovial lining and bone necrosis. MTX and curcumin treated rats showed significant attenuation of the synovial membrane with decreased infiltration of mononuclear cells and reduced pannus growth.

Discussion

The present study demonstrates for the first time, that oral administration of EAD (100, 200 and 400 mg/kg), PF (100 and 200 mg/kg) and CF (100 and 200 mg/kg) produced consistent

antinociceptive, anti-inflammatory and antiarthritic effects in different models of pain and inflammation. Acetic acid causes release of pain mediators, such as prostaglandins and kinins[23]. Oral administration of EAD, and its fraction PF and CF produced a significant anti nociceptive effect in acetic acid induced writhing animals.

Formalin induced pain model is inclusive of two distinctive phases indicating different types of pain[24]. The early phase being neurogenic phase is probably a direct result of stimulation in the paw and reflects centrally mediated pain with release of substance P while later phase (inflammatory phase) involves the release of histamine, serotonin, bradikynin and prostaglandins[25]. In the present study, we found that EAD (400 mg/kg, p.o.), PF (100 and 200 mg/kg, p.o.) and CF (100 and 200 mg/kg, p.o.) were potent enough to block both phases of the formalin response but the effect was more prominent in the second phase. Thus, we suggest that its antinociceptive activity is due to the anti inflammatory action.

The tail flick response is a result of spinal reflex, which is inflected by a supraspinal inhibitory mechanism which exhibits the central mechanism of anti nociceptive[26]. Therefore, EAD and PF might possess centrally and peripherally mediated antinociceptive properties while CF act on peripheral mediated analgesic activity.

Carrageenan induced edema constitutes of two phases, the first phase is mediated by release of histamine and serotonine while the late phase is associated with the neutrophil infiltration, eicosanoid release, production of free radicals and also release of other neutrophil derived mediators[27]. EAD, PF and CF significantly inhibited the paw edema in late phase. Thus, the activity of extract and fractions may be accredited to its ability to inhibit the release of pro-inflammatory mediators mainly, prostaglandin. The cotton pellet induced granuloma model was used to investigate the proliferative phase of inflammation and the EAD, PF and CF produced a significant decrease in granuloma formation that reflected its ability to reduce elevated level of fibroblasts as well as to synthesize collagen with mucopolysaccharide, which are natural proliferative actions of granulation tissue formation[28].

FCA induced arthritis model is associated with destruction of the joints. Paw swelling and arthritic scores are indicative measures to determine antiarthritic activity of any drug[29]. EAD, PF and CF significantly decrease both the indexes as compared to control group in a dose dependent manner.

Hemoglobin and RBCs decrease in RA due to reduced bone marrow erythropoietin response and demolition of premature RBCs[30]. Increased synthesis of endogenous protein such as fibrinogen, α and β globulin and IL-1β mediated increase in the respective colony stimulating factor accounts for increase in the level of ESR and WBCs count. Hence, these parameters form key biomarkers that are regulated during inflammation, stress and cell necrosis[31]. In this study, treatment with EAD, PF and CF in arthritic rats significantly increases the level of Hemoglobin and RBCs whereas it decreases the level of ESR and WBCs which may support its anti-inflammatory potential.

Thymus and spleen are two vital organs involved in the immune response and their relative weights are used as a primary indicator to evaluate the immune regulatory activity. Spleen plays a key role in preventing infection and acting as a first line of defense against invading pathogens by detecting the damaged blood cells and eradicates antigens by phagocytosis[3,32]. The primary role of thymus in the organism is processed immature precursor T-lymphocytes into the mature immune competent T-cells of the medulla[33]. EAD (400 mg/kg, p.o.), PF and Curcumin decreased the spleen index while no effect was observed on thymus index as compared to control group. Therefore, EAD and PF showed their beneficial effect on RA which confirms that they may exhibit antiarthritic activity by an immune suppressant mechanism.

Antioxidant marker enzymes and free radical were evaluated in ankle tissue to inspect the implication of antioxidant potential of extract and its fractions in inflammation. Oxidative stress during inflammation elicits increased reactive oxygen species (ROS) formation due to membrane damage leading to imbalance between antioxidants and free radicals. Elimination of ROS can be achieved by augmenting antioxidant enzymes such as SOD, GSH and CAT or by scavenging the free radicals like LPO[34,35]. EAD, PF and CF significantly revived the deficient SOD, GSH and CAT while reduced free radical LPO level indicating its anti oxidant potential.

TNF-α, IL-6 and IL-1 are pivotal mediators of cell migration and inflammation in RA in which TNF-α have potential to degrade cartilage and bone while IL-1β is abundantly found in the synovial membrane can responsible for joint inflammation as

well as systemic signs of inflammation and IL-6 have a critical role in the pathogenesis of RA, which is responsible for bone resorption and also contributes in the synthesis of auto antibodies such as rheumatoid factor[36]. EAD, PF and CF treated rats markedly decrease the cytokine level in serum and control the progression of RA.

RA conditions were diagnosed by radiography which specifies the severity of disease. In FCA group, ankylosis, osteophytosis, bone erosion and soft tissue swelling were observed in a developed stage of arthritis. Moreover, the protective effect of EAD, PF and CF in progression of joint damage was confirmed by radiological study and they effectively reduced the disease progression in arthritic rats.

Histopathology study provides a perceptible morphological distinctiveness and identified the ability of the bone store-form upon treatment with EAD, PF and CF. The EAD 400 mg/kg dose exhibited potential pharmacological effect and have the ability to suppress inflammation, synovitis and protect the joint.

HPTLC finger printing of EAD and its fractions revealed presence of triterpenoid (ursolic acid and lupeol) and flavonoids (quercetin and kaempferol) which play pivotal role in treatment of inflammation and related complications[25,37]. Several studies have reported that flavonoids exhibits its anti-inflammatory activity by reducing the expression of various pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8 and monocyte-chemoattractant protein-1. Triterpenoids have been demonstrated to elicit anti-inflammatory response attributing to several actions including suppression of lipoxygenase and cyclooxygenase activities, along with restraining of complement activity. Thus, the presence of triterpenoid and flavonoids in EAD may act as a major contributing factor in its observed anti- inflammatory, anti-nociceptive and anti-arthritic activity[38,39].

Conclusion

The present study showed that EAD possessed more significant antinociceptive, anti inflammatory and antiarthritic activities as compared to its fractions PF and CF in a dose dependent manner and EAD (400 mg/kg, p.o.) was more potent than the curcumin. EAD, PF and CF reduced the hind paw swelling and arthritis index. The antiarthritic activity is associated with the ankle histopathological changes, downregulation of cytokine level and radiographic analysis which was evident from the study.

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Conflict of Interest

No conflict of interest has been reported by all the authors.

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