

Triterpenoid fraction isolated from *Euphorbia tirucalli* Linn. ameliorates collagen induced arthritis in Wistar rats

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ABSTRACT

The present study evaluates the anti arthritic efficacy of triterpenoid fraction isolated from *Euphorbia tirucalli* Linn. (TET) in collagen induced arthritis model (CIA). TET at a dose of 100 and 200 mg /kg p.o was tested in CIA induced animals. The degree of inflammation was evaluated by hind paw swelling, arthritic index, body weight and hematological parameters. Radiological and histopathological evaluations of ankle joints were also studied. Oral administration of TET (200 mg/kg) exhibited significant ($p < 0.05$) anti arthritic activity by decreasing the paw volume, normal body weight gain as compared to CIA groups. The altered hematological parameters like C-reactive protein (CRP) and white blood cells level in CIA groups were bring back to near normal in TET (200 mg/kg) treatment group. Further radiological and histopathological studies revealed the anti arthritic activity by indicating remodeling of ankle joints in TET treated groups. The results were parallel with standard drug diclofenac (5 mg/kg). Taken together findings from the current investigation suggest that TET has beneficial anti arthritic effect by reversing the pathological condition in a significant manner.

INTRODUCTION

Arthritis is a chronic inflammatory condition of the bone joints that is associated with hyperalgesia (Ekambaram *et al.*, 2010) and characterized by infiltration of the synovial membrane with T lymphocytes, macrophages and pannus formation over the underlying cartilage and bone subsequently with progressive, erosive destruction of articular tissues and functional impairment (Yoshimura *et al.*, 2014; Yuan *et al.*, 2012). Conventional treatment of arthritis includes Non steroidal Anti-Inflammatory Drugs (NSAIDs), analgesics, glucocorticoids and Disease-Modifying Anti Rheumatic Drugs (DMARDs). Current treatment of arthritis is to minimize the associated pain and inflammation using NSAIDs and decelerate the disease progress by using DMARDs (Patil *et al.*, 2011). However long-term oral intake of these drugs is associated with unpleasant side effects such as gastrointestinal and cardiovascular toxicity (Vetal

et al., 2013). Because of this reason people suffering from chronic inflammatory condition are likely to seek complementary and alternative medicine therapies including herbal drugs for the symptomatic relief. However, despite an increase in use, evidence for effectiveness and safety of these complementary therapies is limited. *E. tirucalli*. (Euphorbiaceae) commonly known as pencil tree, is an ornamental, succulent plant naturally distributed in paleotropical region of Madagascar, Cape region (South Africa), East Africa, but now it has become acclimatized and grows freely in all parts of India (Gupta *et al.*, 2013).

E. tirucalli produces and stores abundant amounts of latex in so-called laticifers and it contains high amounts of sterols and triterpenes (Hastilestari *et al.*, 2013). Traditionally the latex of the plant has been used as an application for warts, asthma, rheumatism, neuralgia, tumors and tooth ache in India (Khaleghian *et al.*, 2011).

E. tirucalli has been reported to present numerous pharmacological activities such as the latex of this plant exhibited strong oxytocic activity against isolated strips of the gravid rat uterus (Mwine *et al.*, 2013), and exhibited microbicidal activity against human pathogens (Prasad *et al.*, 2011).

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It has been demonstrated that the crude latex of *E. tirucalli* modulates the cytokine response of leukocytes, especially CD4⁺ T lymphocytes (Avelar *et al.*, 2011) and it has a promising activity in modulation of myelopoiesis there by enhancing the resistance of tumor-bearing mice (Valadares *et al.*, 2006). However efficacy of triterpenoid fraction isolated from *E. tirucalli* has not been validated for chronic inflammatory conditions. In the current investigation triterpenoid fraction isolated from *E. tirucalli* was evaluated to determine its inhibitory effect on chronic inflammatory response in collagen induced arthritis in wistar rats.

MATERIALS AND METHODS

Chemicals and reagents

Complete Freund's adjuvant and type II collagen was purchased from Sigma Aldrich Co., USA. C-Reactive protein kits were acquired from Diasys Diagnostic kit (Germany). All other chemicals used were analytical reagent grade.

Isolation of Triterpenoid fraction from *E. tirucalli*

E. tirucalli was collected from Karunagappally area of Kollam in March 2014 and confirmed taxonomically by by Rojimon Thomas, Assistant Professor, Department of Botany, C.M.S College, Kottayam. A voucher specimen (voucher No:275) was preserved at Department of Pharmaceutical Science, Cheruvandoor Campus, M.G University, Kottayam district, Kerala. The freshly collected 1000g stem bark of *E. tirucalli* was soaked in 1.5L of 95% of ethanol. After 7 days the extract was filtered and concentrated. Then it was partitioned between ethyl acetate and water. The former fraction was further partitioned into hexane to isolate triterpenoids. The concentrated hexane fraction was then weighed. The percentage yield of hexane fraction of *E. tirucalli* Linn was found to be as 0.984% w/w.

Animals and induction of arthritis

4-8 weeks old male Wistar albino rats with 150-200g purchased from host department animal facility were used for the study. Animals were housed in polypropylene cages at a temperature of 25-30°C and relative humidity 35-45%, in light and dark cycles of 12am and 12pm hour respectively for one week before and during the experiments. All animal care and experimental procedures were in accordance with the guidelines of the animal ethics committee CPCSEA according to Government of India accepted principles for laboratory animals' use and care with IAEC No: 013/MPH/DPS/CVR/14. The rats were randomly allocated into 5 groups with six rats per group as follows: Normal untreated, collagen induced (CIA), CIA+ diclofenac (DFC), CIA+TET 100mg/kg, CIA+TET 200mg/kg. Arthritis was induced by using Chicken Sternal Collagen Type -II with Incomplete Freund's Adjuvant. On day 1, collagen in acetic acid was emulsified with equal volumes of Incomplete Freund's Adjuvant to produce the inducing agent and stored on ice before use. Rats were immunized intradermally with 0.5ml of the emulsion. On day 7, after the primary immunization all animals were given booster

injection with 0.1ml of chicken collagen emulsified with Incomplete Freund's Adjuvant in the same manner.

Measurement of Paw volume

Paw volumes of animals from respective groups were recorded by using digital plethysmometer on day 1, 14, 18, 22, 30, 36 and 40 days after collagen induction. To avoid bias, treatment group was blinded to the investigators performing the assessments.

Arthritic index and body weight change

Rats were examined every week until week 6 after initial sensitization using collagen with Incomplete Freund's Adjuvant. The severity of inflammation in each limb was evaluated every week for the degree of inflammation, the extent of erythema and edema of the periarticular tissues, and the enlargement, distortion, or ankylosis of the joints. Findings were scored on a scale of 0-4, where 0 = no inflammation. 1 = inflammation of 1 joint. 2 = unequivocal inflammation of at least 2 joints of the limb or moderate inflammation of 1 joint. 3 = severe inflammation of 1 joint. 4 = maximum inflammation of ≥ 1 joint in the limb. The arthritic index was calculated as sum of these scores (maximum score = 16). Body weight was measured using digital weighing balance from 1 to 40 days from the day of CIA injection.

Radiological evaluation

The radiology of hind paws of each rats were done under anesthesia using phenobarbitone sodium at a dose of 35 mg/kg intra-peritoneally. Anaesthetized rats were placed on a radiographic box at a distance of 107 cm from the X-ray source. Radiographic analysis of normal and arthritic hind paws was performed by using X-ray machine, with a 48 kVp exposure for 0.5 mAs. A blind and independent assessment of the radiological score was performed by two observers. The severity of arthritis in each rat was determined according to radiological score from 0-3, where; 0 = normal or no tissue swelling or no bone damage, 1 = tissue swelling and edema, 2 = joint erosion, 3 = bone erosion and osteophyte formation.

Histological observation

On day 41, one animal from each group was sacrificed by cervical dislocation and the ankle joints of the hind limbs were excised and fixed in Bouin's fluid, subsequently the specimens were decalcified with 10% EDTA for 7 days, dehydrated and embedded in paraffin blocks. Sections of ankle joints (5µm thick) were cut and mounted on slides and stained using haematoxylin and eosin. Grading of cellular infiltration, synovial hyperplasia, pannus formation, joint space narrowing, and cartilage and bone erosion of the ankle joints was blindly investigated by pathologist.

Haematological evaluation

On day-41 post CIA immunization blood samples were collected from the animals of respective group by retro orbital method. The hematological parameters such as white blood cell (WBC) count and Plasma C-reactive protein (CRP) levels were

estimated in an independent laboratory. WBC's were estimated by following normal laboratory procedures. Plasma CRP level was measured by using Diasys Diagnostic kit (Germany).

Statistical analysis

The results obtained are presented as mean \pm standard error. All the statistical analyses were carried out by Graphpad prism version 6.0 using One-way ANOVA followed by Tukey's multiple comparison tests. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of TET on inhibition of paw volume

Paw volumes of the animals in all groups were recorded on day1, 14, 18, 22, 28, 35 and 40. Data obtained were depicted in figure 1 and from the results it is clear that, arthritic animals treated with TET exhibited significant reduction in paw volume.

Group 5 (TET (200mg/kg)) produced a highly significant reduction in the paw volume ($p < 0.05$) was observed on day 40 as compared to the arthritic control (Group I). Group 2 animals (treated with 5mg/kg of standard drug DFC) also produced a similar decrease in paw volume on day 40 ($p < 0.05$) as compared to control animals (Group 1).

Effect of TET on arthritic index and body weight

Arthritic index and body weight of animals in all groups were recorded on day 1, 7, 14, 21, 28, 35 and 40. Data obtained were depicted in figure 2. From the above data it is clear that, arthritic animals treated with TET (Group 4&5) at a dose of 100 & 200mg/kg, produced a highly significant reduction in the arthritic index ($p < 0.001$) on day 40 as compared to the arthritic control (Group I). Moreover treatment with TET 200mg/kg significantly improved ($p < 0.001$) body weight as compared to CIA group (figure 3).

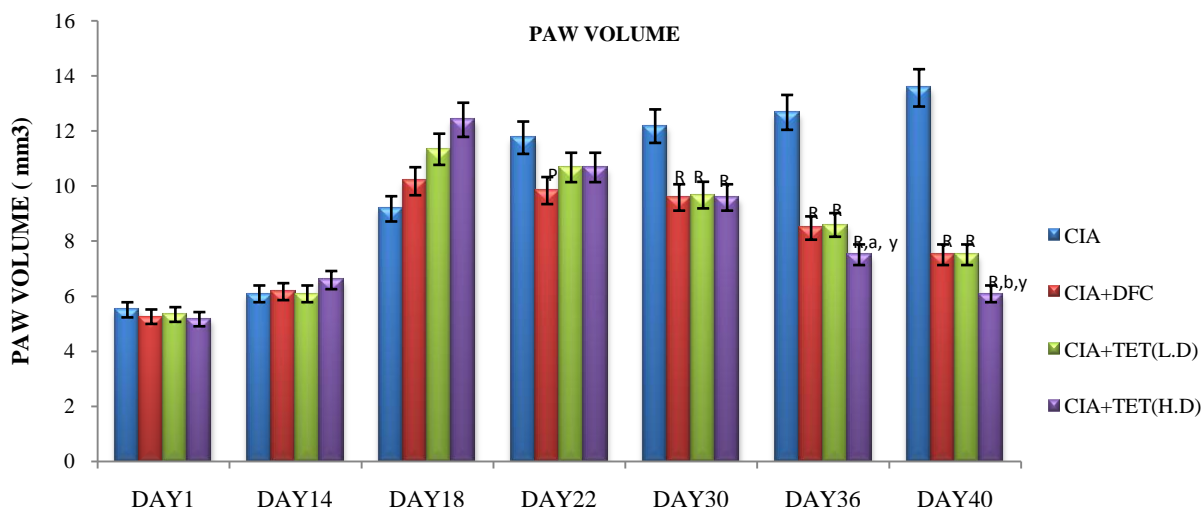


Fig. 1: Effect of TET on mean paw volume of collagen induced arthritic rats. Each bar represent the mean \pm S.E.M, n=6, ^{PQR} represents statistical significance vs. positive control, : ^P $P < 0.05$, : ^Q $P < 0.01$, : ^R $P < 0.001$ as compared to positive control. ^{a,b} represent statistical significance vs standard. ^{a,b} represents statistical significance vs standard group ^a: $P < 0.05$, ^b: $P < 0.01$. ^{x,y} represents statistical significance vs low dose ^x: $P < 0.05$, ^y: $P < 0.01$

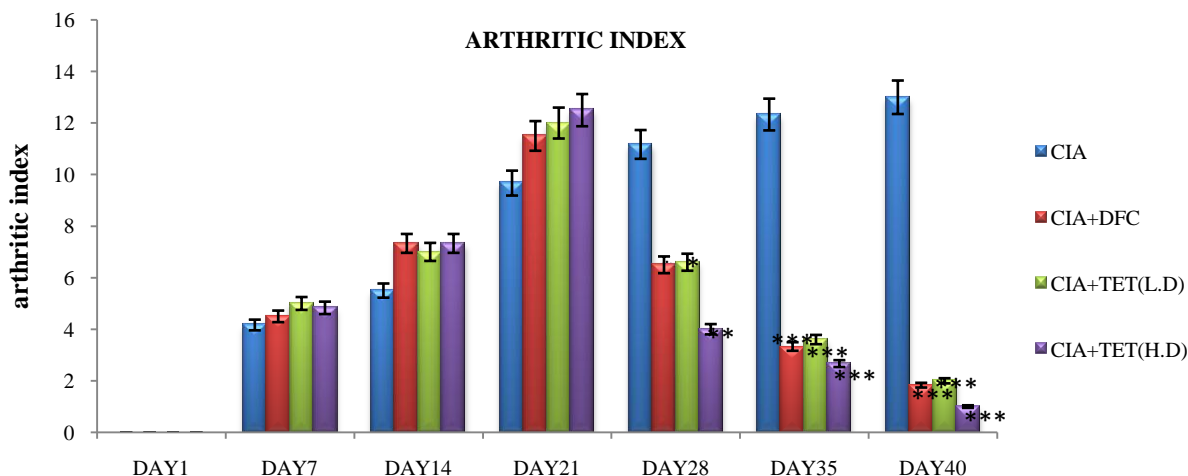


Fig. 2: Effect of TET on mean arthritic index of collagen induced arthritic rats. Each bar represent the mean \pm S.E.M, n=6, *represents statistical significance vs. positive control, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ as compared to positive control.

Effect of TET on radiological evaluation

Radiological scores of animals in all groups were recorded on day 40. Radiological scores were assessed and tabulated in table 1. Radiograph of each group (figure 4) represents 1) normal animal, 2) DFC (5mg/kg) exhibited the cartilage destruction and soft tissue swelling. 3 and 4 represents TET 100 mg/kg and 200 mg/kg treated group showed improvement in articular texture. 5) represents CIA group, exhibited bone destruction and tissue inflammation.

Table 1: Effect of TET on radiological score.

GROUP	CIA	CIA+DFC	CIA+TET (L.D)	CIA+TET (H.D)
MEAN± S.E.M	3±0	1.5±0.2236	1.6±0.2108	0.67±0.1247

Effect of TET on WBC and CRP level

Hematological parameters such as WBC count and CRP level were evaluated on 41th day (figure 5, 6). The WBC count and CRP level was gradually increased in CIA group (8.117 ± 0.162 ; 8.6 ± 0.238) when compared to normal animal

(5.2 ± 0.1528 ; 1.28 ± 0.04014). However TET 200 mg/kg treatment significantly reduced WBC count and plasma CRP level (5.8 ± 0.05774 ; 1.53 ± 0.0614) when compared with CIA group.

Effect of TET on histopathology

Histopathological evaluation of hind paws (Figure 7) joints of normal group rats showed intact articular cartilage and normal synovial lining with absence of inflammation and infiltration of inflammatory cells.

Cartilage damage, synovial thickening, congestion hemorrhage, infiltration of large number of lymphocyte, neutrophils, plasma cells and pannus formation were observed in CIA induced rats.

The standard diclofenac has also maintained normal articular cartilage with reduced infiltration of inflammatory cells as compared to arthritis control group. However both high dose and low dose TET treated group has shown significant protection in inflammatory condition at synovial membrane with normal joint space and scanty lymphocyte infiltration.

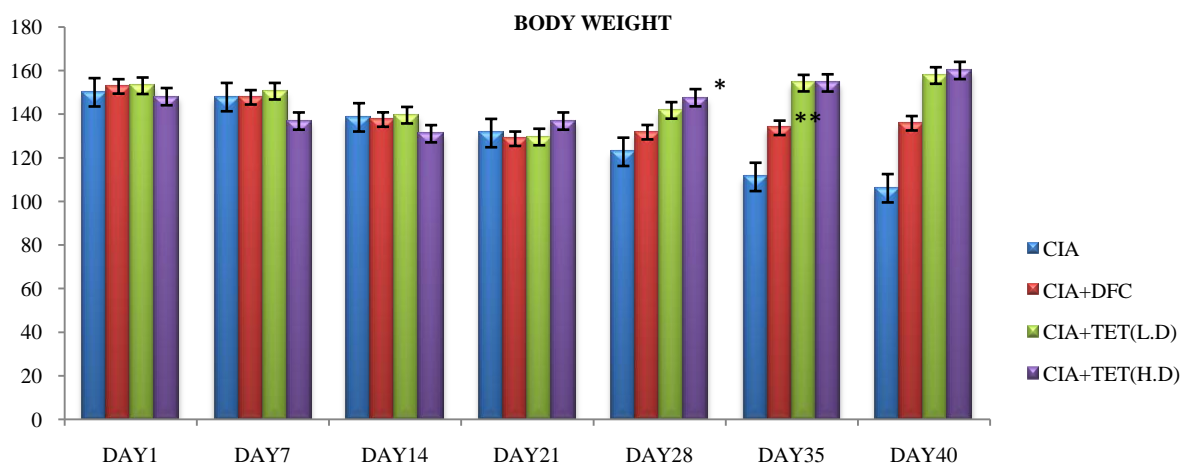


Fig. 3: Effect of TET on mean weight of collagen induced arthritic rats: Each bar represent the mean± S.E.M, n=6, *represents statistical significance vs. positive control, *: P<0.05, **:P<0.01, ***: P<0.001 as compared to positive control. ^{a,b} represent statistical significance Vs standard. ^{a,b} represents statistical significance vs standard group ^a: P<0.05, ^b: P<0.01.



Fig. 4: Radiograph of ankle joints of experimental animals: 1) normal animal, 2) DFC (5mg/kg), 3) TET 100 mg/kg treated group, 4) TET 200 mg/kg treated group, 5) CIA group.

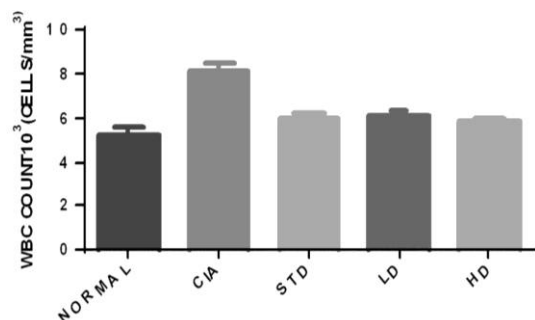


Fig. 5: Effect of TET on WBC count. Values expressed as average of 6 samples \pm SEM

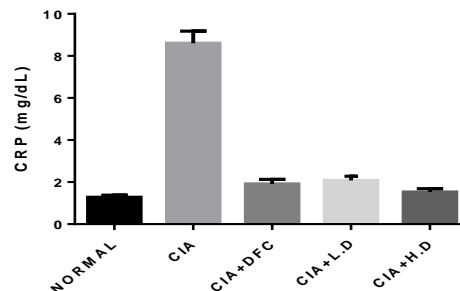


Fig. 6: Effect of TET on CRP level. Values expressed as average of 6 samples \pm SEM

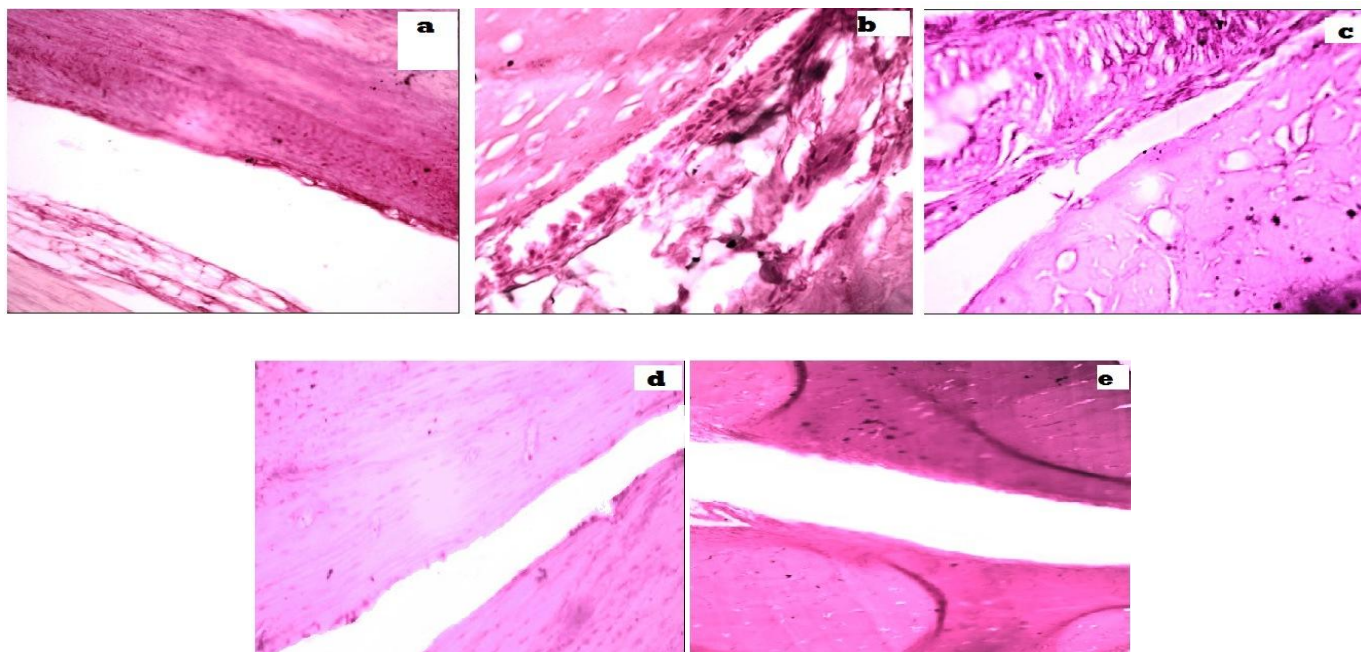


Fig. 7: Histopathology of ankle joints (H&E, 5 μ m, 40X). (a) Denotes normal ankle joint, (b) denotes positive control (CIA) model, (c) denotes diclofenac treated group (CIA+DFC), (d) denotes extract with low dose treated group (CIA+TET(100 mg/kg)), (e) denotes extract with high dose treated group (CIA+TET(200 mg/kg)).

DISCUSSION

Triterpens are ubiquitously distributed in the plant and marine animal kingdom (Alqahtani *et al.*, 2013). They are produced in plant as secondary metabolites and have diverse pharmacological activities such as anticancer, immunomodulatory, anti anxiety as well as antinociceptive. It is well recognized that herbal products containing triterpenes have long been used in many Asian countries to treat or prevent variety of disease by the traditional healers (Parmar *et al.*, 2013). The latex of *E. tirucalli* comprises triterpens are the major constituent (Hastilestari *et al.*, 2013). In the present study we investigated the modulatory effect of triterpenoid fraction isolated from *E. tirucalli* in collagen induced chronic arthritis model. The collagen-induced arthritis model is the most commonly studied autoimmune model of chronic inflammatory conditions like rheumatoid arthritis. The CIA model has been used extensively to identify potential

pathogenic mechanisms of autoimmunity, including the role of individual cell types in disease onset and progression, as well as to design and test new therapeutics. Recently CIA model has been implemented in the testing and development of the new biologically based therapeutics, such as those that target TNF- α , a cytokine produced by macrophages and T cells that is a dominant inflammatory mediator in the pathogenesis of RA (Brand *et al.*, 2007). CIA is elicited in experimental animals by immunization with type II collagen emulsified in complete Freund's adjuvant (CFA), leading to the initiation of a chronic inflammatory response. The ensuing pathogenesis of polyarthritis is associated with immune complex deposition on articular surfaces, and shares several pathological features with RA, including synovial hyperplasia, mononuclear cell infiltration, cartilage degradation, bone resorption and periosteal proliferation, and periarticular inflammation (Bendele *et al.*, 1999). Paw volume and arthritic index is a measure of clinical assessment of joint swelling. The

determination of rat paw swelling is apparently simple, sensitive and one of the quick procedures for evaluating the degree of inflammation and the therapeutic effects of drugs. Paw swelling of periarticular tissues such as ligaments and joint capsules are commonly associated with arthritis (Kshirsagar *et al.*, 2014). The chronic inflammation involves the release of number of mediators like cytokines, interferons etc. These mediators are responsible for the pain, destruction of bone and cartilage that can leads to severe disability (Ghelani *et al.*, 2013). In the current investigation TET (200mg/kg) exhibited a statistically significant reduction of the paw volume when compared with CIA induced group. Rheumatoid arthritis is associated with weight loss and loss of lean body mass, known as rheumatoid cachexia. Rheumatoid cachexia is thought to be end result of cytokine driven hypermetabolism and is a key comorbidity in RA (Roubenoff *et al.*, 1992). CIA rats had shown a steady increase in body weight till day 5. But from day 7, all rats showed a decrease in weight due to the induction of arthritis. The body weight of CIA rats decreased due to severe arthritis. But TET and DFC treated animals exhibited a significant increase the body weight as compared to CIA group. There is increasing evidence that acute phase protein CRP and WBC has a precise role in the inflammatory condition. It has been reported that in arthritis condition a, moderate rise in the WBC count occurs due to an IL-1B mediated inflammatory response, which enhance the production of both granulocyte and respective colony-stimulating factors (Patil *et al.*, 2011; Rajendran and Krishnakumar, 2010). Abnormal rise of plasma CRP might be primarily due to the increased activation of proinflammatory cytokines such as IL-1 and TNF- α in combination with IL-6 (Ghelani *et al.*, 2013). Diminished CRP level in serum of treatment group indicates that TET may inhibit pro inflammatory cytokines such as IL-1, IL-6 and TNF- α . In addition the effect of TET on severity of CIA was further investigated by radiological and histopathological analysis. Radiological evaluation of ankle joint of each group revealed that treatment with TET 200 mg/kg and DFC inhibited the arthritis associated joint destruction. Typical microscopic features present in RA are pannus formation, cellular infiltration and synovial hyperplasia. In the joints of TET administered rats, pannus formation, cartilage destruction as well as cellular infiltration was found to be significantly reduced. In addition, normal joint space between two adjacent articulate or less synovium hyperplasia was observed in TET treated group when compared to CIA induced group. In conclusion our findings indicates that TET was able to attenuate the chronic inflammatory response. Moreover TET at a dose of 200mg/kg could normalize the altered paw volume, haematological and histological changes. Taken together, triterpenoids from *E. tirucalli* may be used as a potent natural anti-inflammatory therapeutic agent for the treatment of arthritis like disorders.

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