Original Article

Anti-arthritic activity of *tridax procumbens* ethanolic extract of leaves

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ABSTRACT

The present study was carried out to evaluate the Antiarthritic activity of *Tridax procumbens* leaves extract in complete Freud's adjuvant (CFA) induced arthritis in rats. Arthritis was induced by injecting 0.1 of CFA in metatarsal footpad of male wistar rats. Degree of arthritis was evaluated by hind paw swelling, body weight, histopathology of knee joint and various other Physiological and Haematological parameters. *Tridax procumbens* ethanolic extract (TPEE) at the dose of 300 mg/kg shows reduction in the pathophysiological conditions as compared to the standards Cyclophosphamide (8 mg/kg) and Diclofenac sodium (10 mg/kg) compared statistically with Tukey's multi comparison test using Graph pad prism. Further detailed investigations were required to isolate the compound responsible for the Antiarthritic activity.

Keywords: Antiarthritic activity, Physiological, Haematological, Knee joint Histopathology, *Tridax procumbens.*

INRODUCTION

Rheumatoid arthritis (RA) is a common chronic and systemic autoimmune disorder characterized by inflammation of the synovial joints and concomitant destruction of cartilage and bone. Affecting the synovial joints and typically producing symmetrical arthritis that leads to joint destruction, which is responsible for the deformity and disability. The proinflammatory cytokines, mainly TNF-α, IL-1β and IL-6 produced by monocytes, macrophages and synovial fibroblasts are suggested to play an important role in the pathogenesis and disease progression of RA.[1] *Tridax procumbens* L. (Compositae) is a hispid, procumbent herb, found as a weed throughout India. *Tridax procumbens* is a perennial plant, 15–40cm high, often rooting at the nodes, sometimes developing a woody base.[2] Its common names include coat buttons and tridax daisy in English, cadillo chisaca in Spanish, herbe caille in French, jayanti veda in Sanskrit, ghamra in Hindi, bishalya karani in Oriya, dagadi pala in Marathi, gaddi chemanthi in Telugu, thata poodu in Tamil and kotobukigiku in Japanese.[3] The poultice of whole plant is used as an anti-inflammatory remedy by the tribal healers.[4] The previous pharmacological investigations on *Tridax procumbens* reported to posses various activities like Analgesic, Antipyretic,[5] Acute Anti-inflammatory,[6] Immunomodulatory,[7] Wound healing,[8] Hepatoprotective[9,10] Anti-leishmanial[11] Anti-hyperglycemic,[12] Anti-diabetic,[13] Antimicrobial[14] and Hair growth promoting activities.[15] The Phyotochemical investigation reports the isolation of lipid constituents, sterols, flavonoids, polysaccharide and bergenin derivatives from *T. procumbens*.[16,17] The leaves are reportedly used to treat bronchial catarrh dysentry and diarrhoea and as a hair restorative. In southern orissa a paste prepared from the whole plant is taken orally to relieve diarrhoea. A fine paste of the leaves is applied externally to reduce swelling of hemorrhoids. The whole plant and seed being used to treat a variety of ailments the leaves are cooked and eaten as a vegetable.[18]

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The designed work can be rationalize as, Antioxidant[4] property of *Tridax procumbens,* Activity against Analgesia, Pyrexia[5] and Inflammation[6] as well as presence of sterols and flavanoids provide a view that the active constituents of *Tridax procumbens* can also have activity against arthritis, hence the aim of present study is to evaluate the *in- vivo* antiarthiritic activity of *Tridax procumbens* leaf extract by adjuvant arthritis assays in rats.

MATERIAL AND METHODS

The plant was collected in the month of September-October 2010 from the fields of village Mau, Dist Narsinghpur (M.P). It was authenticated by Prof. A.B.Tiwari, Head of Department of Crop and Herbal Physiology, Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur (M.P.) and the specimen voucher no. assigned was DCHP/1610/Sep10/JNKV. The leaves were dried under shade and pulverized to coarse powder which was passed through sieve No.20 to maintain uniformity and further extracted with 90% ethanol and ethyl acetate by hot extraction process using soxhlet apparatus at 55–60ºC for 35 complete cycles.[7]

Animals care and handling

The experiment was carried out on wistar albino rats of either sex of weight 150 ± 20 grams. They were provided from Truba Institute of Pharmacy, Bhopal and were acclimatized to the standard laboratory conditions in well cross ventilated animal house at temperature 25 ± 2°C relative humidity 44–56% and light and dark cycles of 13 and 11 hours respectively for 1 week before and during the experiment. They were fed with standard diet (Golden Feeds, Delhi) and water *ad libitum*. The experiment was approved by the Institutional Animal Ethics Committee and as per CPCSEA guidelines (approval no. 1196/a/08/CPCSEA).

Drugs and chemicals

Diclofenac sodium provided by Novartis pharmaceuticals Pvt. Ltd, Cyclophosphamide provided by Cadila healthcare Ltd., Complete Freund's adjuvant (Sigma) and RA test kit (Span) were purchased and all other chemicals utilized were of analytical grade.

Acute toxicity studies

The LD_{50} was calculated by "Staire case" method. The LD_{50} was determined in rats and mice by oral and intraperitoneal route. The initial dosing was 2000 mg/kg p.o. and 800 mg/kg i.p. in both the species.[5] On the basis of above study the dose 300 mg/kg P.O. was selected for the further study.

Preparation of extract dose

2% acacia suspension was prepared by suspending 2 gram of accurately weighed acacia powder in 100 ml of 0.9% saline. 20 ml of vehicle was taken separately to which 2 gram of dried extract was added and sonicated, this produce suspension of 100 mg/ml strength. Both *Tridax procumbens* ethanolic extract (TPEE) and *Tridax procumbens* ethyl acetate extract (TPEAE) suspension were prepared in such manner.

Animal grouping

Screening of Anti-arthritic activity in rats by Adjuvant arthritis model

Physiological screening

On day 0, 0.1 ml of complete Freund's adjuvant was injected into the sub plantar region of the left hind paw of all the animals. Dosing with the test and the standard drugs were started on the same day and continued for 14 days. From day 14 to 21 the animals were not dosed with the test compound and the standard drugs. Body weights of animals of all the groups were periodically monitored by using electronic balance on 0th 7th, 14th and 21st day. The paw was marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark and measured plethysmographically (a U shaped instrument filled with mercury held upto a fixed height) immediately after injection and on 7th, 14th and 21st day. No. of nodes were also monitored at the same interval.[19,20]

Haematological screening

On 21st day the animals were sacrificed as per the norms of CPCSEA guidelines by the physical method of euthanasia (cervical dislocation) and their blood sample was collected for testing various haematological parameters such as WBCs, RBCs, ESR, Hb and RF. ESR

Table 1: Animal grouping and their drug administration protocol

Group No.	TREATMENT	DOSE
I (Negative control)	CFA + Vehicle	0.1 ml(SP) + 1 ml/100gm/P.O.
II (Standard 1)	CFA + Diclofenac sodium	0.1 ml(SP) + 10 mg/kg I.P.
III (Standard 2)	CFA + Cyclophosphamide	0.1 ml(SP) + 8 mg/kg I.P.
IV (Test 1)	CFA +TPEE	0.1 ml(SP) + 300 mg/ kg P.O.
V (Test 2)	CFA + TPEAE	0.1 ml(SP) + 300 mg/ kg P.O.

CFA- Complete Freund 's adjuvant, TPEE-*Tridax procumbens* ethanolic extract, TPEAE- *Tridax procumbens* ethyl acetate extract, SP- sub plantar, I.P.-Intra peritoneal, P.O. - Per oral.

was estimated by using Westergern tube having range of graduation 0–200 mm, height of blood column 200 mm, length 300 mm, internal diameter 2.5 mm. One ml blood sample was filled in the tube previously fused with anticoagulant solution and allowed to fix vertically in sedimentation rate rack for a hour.[21] WBCs were counted by sucking blood sample upto 0.5 mark in the WBC diluting pipette and holded horizontally, diluted using WBC diluting fluid upto 100 mark and shaked for 10 seconds, counting chamber was charged, 2–3 drops placed on the neuber slide and investigated under medical microscope.[21] RBCs were counted by sucking blood sample in RBC diluting pipette upto 0.5 mark and holded horizontally, and then RBC diluting fluid was taken upto mark 100, counting chamber was charged, sample was placed on neuber slide and investigated under medical microscope.[21] Haemoglobin determination involves determination of percentage of haemoglobin present in blood sample. Numbers of method are available for its estimation among which colorimetric method was used by using visual haemoglobinometer. The visual method of Hb determination is called as Shali-Hellige Method. Graduated tube was filled upto mark with 0.1N HCl then 20 cu mm of blood sample was added in it. Then the content of graduated tube was diluted until the colour of the solution matches with the standard colour strips. Sedimented volume in the tube was observed.[21] RF test was carried out by using RA Test Kit. Blood sample of animals were collected, serum separated out. Positive control serum was spotted on two circles of RA test slide. The standard antigen (RA test antigen, consist of polystyrene latex particles with specially purified human gammaglobulins). Sample serum was placed on the separate circles of RA test slide by using the disposable plastic droppers, mixed well with a disposable applicator stick not more than two minutes and the agglutination was examined. Sample of all animals in each group were examined. Test was interpretated as follows; Coarse agglutination (usually occurs within one minute) - Strongly positive, Finer agglutination- Weakly positive, Smooth suspension, without any major change (usually takes full two minutes) - Negative.[22]

Knee joint Histopathology

On the 21st day the animals were sacrificed as per the norms of CPCSEA guidelines by the physical method of euthanasia (cervical dislocation). The hind paws were dissected out. Fragments of tarso-metatarsal joints were collected and fixed in 10% buffered formalin. The fragments were then treated with a 10% acidic nitric solution for decalcification, dehydrated, cleared, embedded in paraffin, 3–4 mm thick sections were cut and stained with Haematoxylin and Eosin. Synovial inflammation, juxta-articular erosion, accumulation of neutrophils, granulamotous tissue, tendon and skeletal muscle inflammation were observed. The joints of at least three animals were observed in each experimental group.[23]

Statistical Analysis

The results were expressed as mean ±SEM. The results of the present study were analyzed using one way ANOVA followed by Tukey's multiple comparison test computed statically by using Graph pad prism software (version 5.04) at $p<0.05$.

RESULTS

The physiological parameters (Body weight, No. of nodes and Paw volume) were monitored on 0th,7th, 14th and 21st day among them the body weight continuously decline throughout the treatment majorly in group I while in the remaining groups overall no gain no loss was observed. Paw volume of all the animals in each group increase initially but decrease after treatment with standard and test drugs; but in vehicle treated remain increased. Nodes does not appeared in the initial phase of inflammation but with the progression of disease they appeared in the various parts of body such as hind paws, tail, fore paws, ears but not in nose. Maximum numbers of nodes were found on 16th day in the vehicle treated group while minimum in Cyclophosphamide treated group. On interpretation of the data of physiological parameters *Tridax procumbens* ethanolic extract at 300mg/kg showed significant ($P<0.001$) while 300mg/kg ethyl acetate extract of *Tridax procumbens* showed less significant results comparing with group I while both were less and not significant with II and III group respectively by One way ANOVA followed by Tukey's multiple comparison test (Table No.1). The hematological parameters such as RBC, WBC, ESR, RF and Hb were observed on the 21st day by collecting the blood sample of animals of all groups. RBCs and Hb of group I and III were found less than normal range while of rest groups were nearby normal while the total leucocytes count (WBCs) and ESR were less than normal in animals of group I and nearby normal range in drug treated groups. *Tridax procumbens* ethanolic extract showed better results than ethyl acetate extract at 300mg/kg comparatively; as TPEE showed significant ($P<0.001$–0.05) whereas TPEAE was less significant ($P<0.05$) comparing with various groups by One way ANOVA followed by Tukey's multiple comparison test. The Rheumatoid factor was found negative in animals of all groups (Table 2). The observed histopathological changes under medical microscope at 10X and than photomicrography of knee joints of the experimental

Table 2: Effects of TPEE and TPEAE on Paw volume against CFA induced arthritis in rats.

	Data of Paw Volume in ml (Mean ± SEM)			
GROUP	**DAY 0**	**DAY 7**	**DAY 14**	**DAY 21**
I	0.22 ± 0.011	0.86 ± 0.058	0.93 ± 0.064	0.86 ± 0.083
II	0.24 ± 0.013	0.36 ± 0.088^{a**}	0.36 ± 0.105^{a**}	0.30 ± 0.089^{a**}
III	0.25 ± 0.016	0.31 ± 0.097^{a**}	0.37 ± 0.096^{a**}	0.075^{a**}
IV	0.20 ± 0.010	0.38 ± 0.090^{a**}	0.35 ± 0.086^{a**}	0.39 ± 0.092^{a*}
V	0.23 ± 0.010	0.43 ± 0.091^{a*}	0.42 ± 0.091^{a**}	0.45 ± 0.011^{a*}

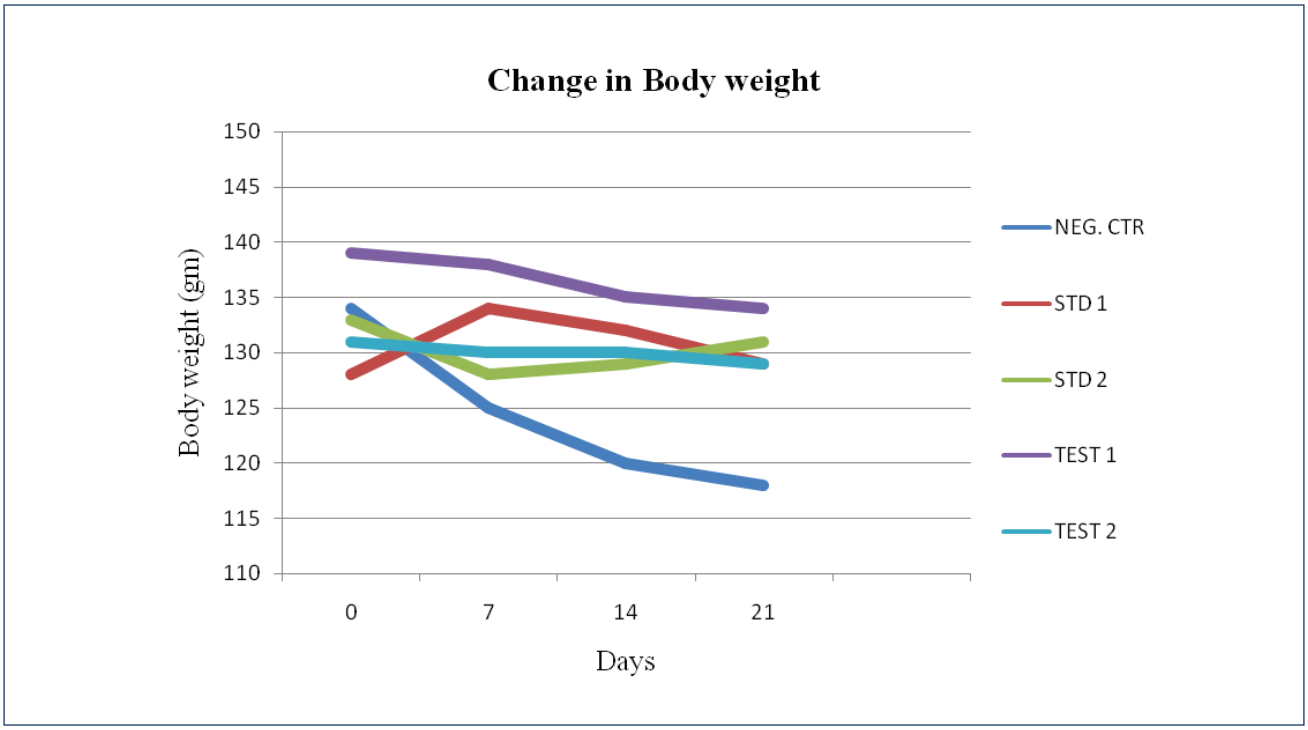
N = 6, Data expressed as mean ± SEM, One way ANOVA followed by Tukey's multiple comparison test.
a-comparison with group I(negative control) of all groups, b-comparison with Group II(Std. 1) of IV & V Groups
c-comparison with Group III(Std.2) of IV & V Groups,***P<0.001, ** P<0.01, * P<0.05.

Table 3: Effects of TPEE and TPEAE on Hematological Parameters against CFA induced arthritis in rats.

GROUPS	**RBC (million/mm^3) Mean±SEM**	**WBC (thousand/mm^3) Mean±SEM**	**ESR (mm/hr) Mean±SEM**	**HB (g/dl) Mean±SEM**	**RF**
I	4.65 ± 0.31	11.83 ± 1.13	11.9 ± 1.32	10.7 ± 0.53	–ve
II	7.5 ± 0.40^{a***}	7.2 ± 0.86^{a**}	5.33 ± 1.25^{a**}	13.67 ± 0.66^{a*}	–ve
III	5.5 ± 0.27^{b**}	7.33 ± 0.61^{a**}	4.90 ± 0.84^{a***}	9.83 ± 0.87^{b**}	–ve
IV	6.4 ± 0.21^{a**}	8.2 ± 0.48^{a*}	5.51 ± 0.73^{a**}	12.67 ± 0.30^{c*}	–ve
V	6.1 ± 0.40^{a*}	9.38 ± 0.33	6.36 ± 0.80^{a**}	11.5 ± 0.67^{c*}	–ve

No. of animals in each group = 6, Data expressed as mean ± S.E.M, One way ANOVA followed by Tukey's multiple comparison test. a comparison with group I (negative control) of all groups, b comparison with Group II(Std. 1) of IV & V Groups., c comparison with Group III(Std.2) of IV & V Groups, ***P<0.001, **P<0.01, * P<0.05

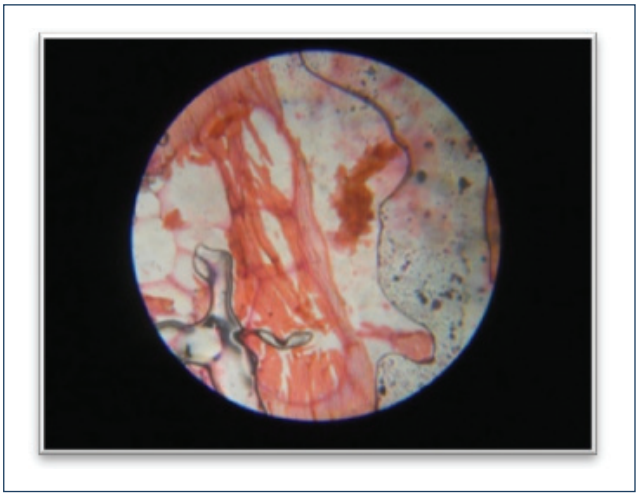
groups are shown in Figure 1–5. Group I showed the histopathology of negative control arthritic rat joint showed prominent abnormalities from the normal joint like edema formation, degeneration with partial erosion of the cartilage, destruction of bone marrow and extensive infiltration of inflammatory exudates in the articular surface Severe periarticular inflammation with marked edema was present around areas of bone, bone destruction, synovial membrane depletion as well as periosteal proliferation. The Diclofenac sodium (group II) treated rat joint showed mild boney destruction prompt synovial membrane depletion, mild surrounding tissue injury but no periosteal proliferation. The Cyclophosphamide treated (group III)



Graph 1: Effects of TPEE and TPEAE on Body Weight against CFA induced arthritis in rats.

rats joints showed normal bone structure mild periarticular inflammation with nil edema around areas of bone, bone destruction negligible, synovial membrane depletion negligible as well as no periosteal proliferation with less cellular infiltrates. TPEE treated group showed better histology than TPEAE treatment for 21 days as there were less inflammatory signs like scanty cellular infiltrate, absence of edema formation and normal done along with synovial membrane.

Histopathological analysis



Group I Negative control (CFA + Vehicle treated) Photomicrograph of knee joint from adjuvant arthritic rat 21 days post-adjuvant injection at the sub plantar region of left hind paw. Severe periarticular inflammation with marked edema is present around areas of bone, bone destruction, synovial membrane depletion as well as periosteal proliferation (magnification= 10X).

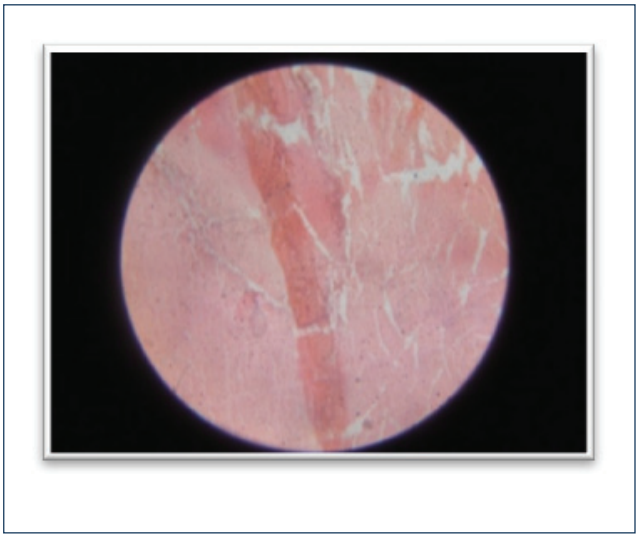


Figure 2: Group II STD. 1 (CFA + Diclofenac sodium treated) Photomicrograph of knee joint from adjuvant arthritic rat 21 days post-adjuvant injection at the sub plantar region of left hind paw. Mild periarticular inflammation with nil edema around areas of bone, boney destruction not seen, prompt synovial membrane depletion, mild surrounding tissue injury but no periosteal proliferation (magnification= 10X).

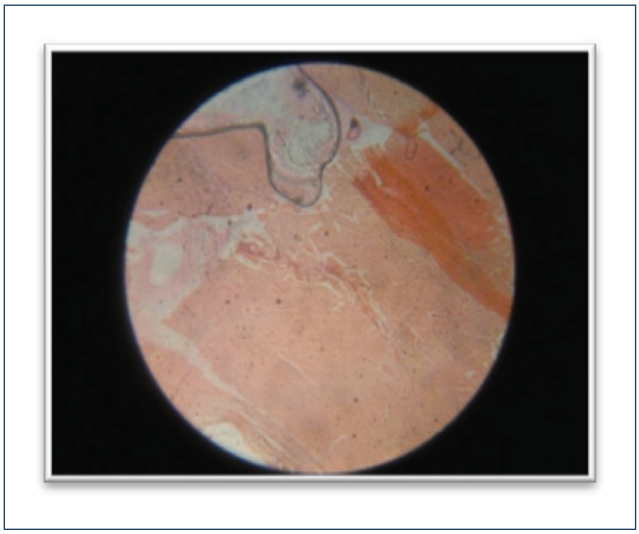


Figure 3: Group III STD. 2 (CFA + Cyclophosphamide treated) Photomicrograph of knee joint from adjuvant arthritic rat 21 days post-adjuvant injection at the sub plantar region of left hind paw. Mild periarticular inflammation with nil edema around areas of bone, bone destruction negligible, synovial membrane depletion negligible as well as no periosteal proliferation (magnification= 10X).

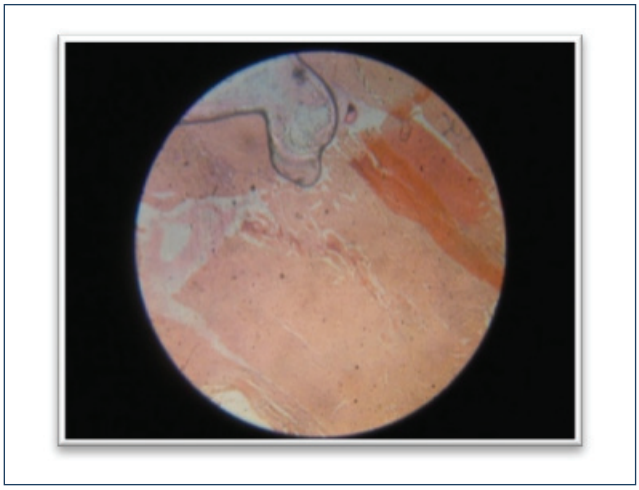


Figure 4: Group IV Test 1 (CFA + *Tridax procumbens* ethanolic extract treated) Photomicrograph of knee joint from adjuvant arthritic rat 21 days post-adjuvant injection at the sub plantar region of left hind paw. Mild periarticular inflammation with nil edema around areas of bone, boney destruction not seen, mild synovial membrane depletion, prompt surrounding tissue injury but no periosteal proliferation (magnification= 10X).

DISCUSSION

Rat adjuvant arthritis is an experimental model of polyarthritis which has been widely used for preclinical testing of numerous anti-arthritic agents which are either under preclinical or clinical investigation or are currently used as therapeutics in this disease. The hallmarks of this model are reliable onset and progression of robust, easily measureable, polyarticular inflammation, marked bone resorption and periosteal bone proliferation. Cartilage destruction occurs but is disproportionally mild in comparison to the inflammation and bone

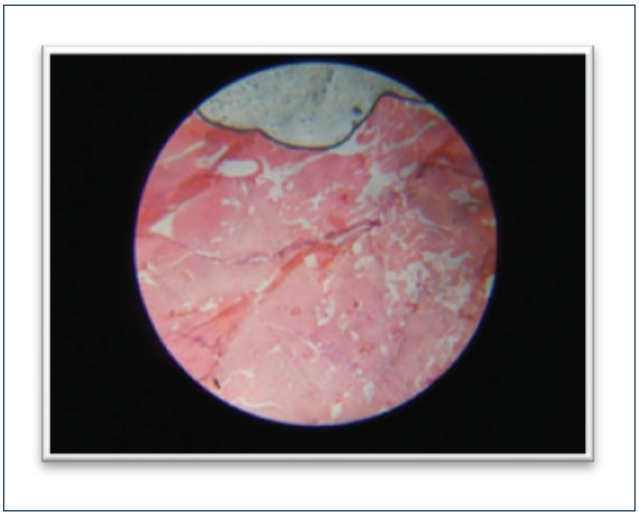
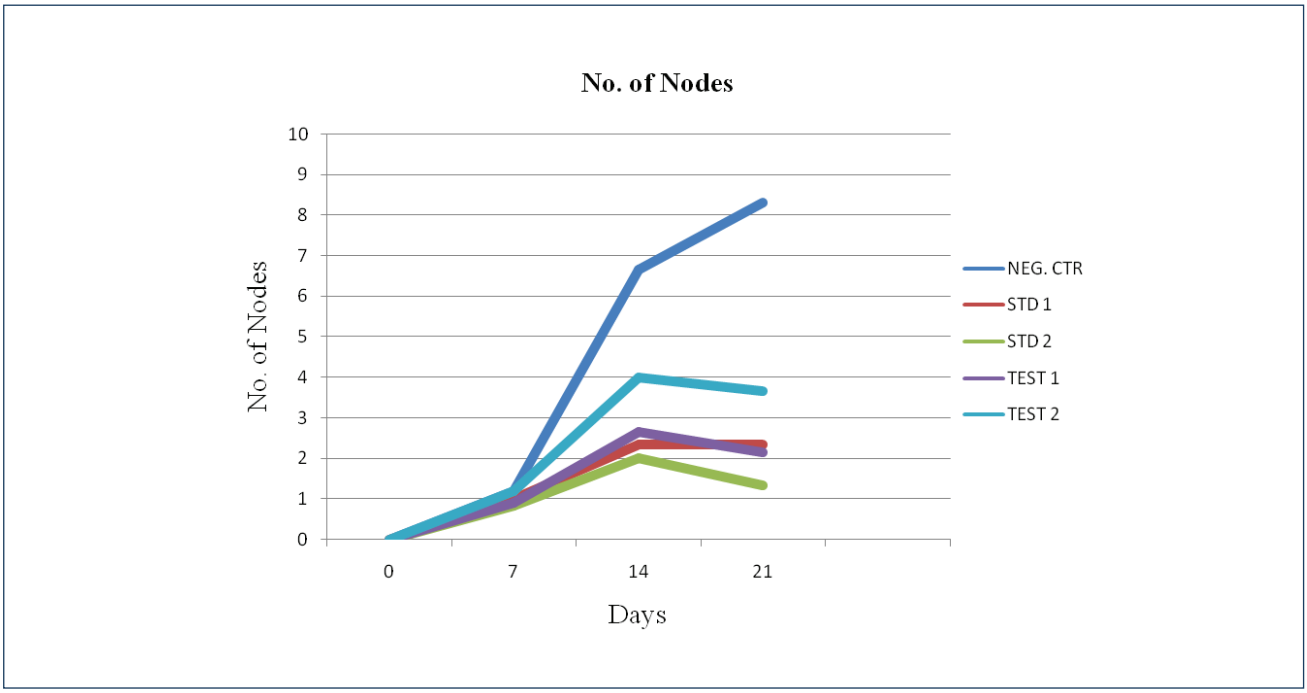


Figure 5: Group V Test 2 (CFA + *Tridax procumbens* ethyl acetate extract treated) Photomicrograph of knee joint from adjuvant arthritic rat 21 days post-adjuvant injection at the sub plantar region of left hind paw. Mild periarticular inflammation with nil edema around areas of bone, boney destruction, synovial membrane depletion, surrounding tissue injury but no periosteal proliferation (magnification= 10X).

destruction that occurs.[20] The parameters assessed in the anti arthritic study were physiological parameters, haematological parameters and histopathological study of knee joints. Complete Freund's adjuvant was used to induce arthritis in rats; 0.1ml of it was injected in the sub plantar region of the left hind paw considered as day 0, treatment started on the same day till 14th day. The animals of all groups subjected to their respective treatment to evaluate the prophylactic use of test drug.

In the present study, the rats were selected to induce arthritis because rats develop a chronic swelling in multiple joints with the influence of inflammatory cells, erosion of joint cartilage and bone destruction. It has close similarities to human rheumatoid disease.[24] These mediators are responsible for the pain, destruction of bone and cartilage that can leads to severe disability.[25] However, the standard drug Diclofenac sodium, Cyclophosphamide and the ethanolic extract significantly suppressed the swelling of the rat paws. In arthritic condition, there is a mild to moderate rise in WBC count due to the release of IL-IB inflammatory response, IL-IB increases the production of both granulocyte and macrophages colony stimulating factors.[25,26] In the present study, the migration of leucocytes into the inflamed area is significantly suppressed by TPEE when compared to standard drug Diclofenac sodium, Cyclophosphamide, as seen from the significant reduction in the total WBC count. Erythrocyte Sedimentation Rate (ESR) is an estimate of the suspension stability of RBC's in plasma. It is related to the number and size of the red cells and to the relative concentration of plasma proteins, especially fibrinogen, alpha and beta globulins. Increase in the rate, is an indication of active but obscure disease processes. The acute phase proteins in ESR and C-Reactive Proteins (CRP) share the property of showing elevations in the concentration in response to stress or inflammations like injection, injury, and surgery and tissue necrosis. The ESR count significantly increased in arthritic control group, whereas these counts were remarkably



Graph 2: Effects of TPEE and TPEAE on Number of Nodes against CFA induced arthritis in rats.

counteracted in the standard Diclofenac sodium, Cyclophosphamide and ethanol extract groups and thus justifying its significant role in the arthritic conditions.[26] Changes in body weight have also been used to assess the course of the disease and the response to therapy of anti-inflammatory drugs.[27] As the incidence and severity of arthritis increased, the changes in the body weights of the rats also occurred during the course of the experimental period. The loss of the body weight during arthritic condition was also supported by earlier observations,[28] on alterations in the metabolic activities of diseased rats. Earlier findings suggest that absorption of 14C-glucose and 14C-leucine in rat's intestine was reduced in the case of inflamed rats.[29] Treatment with anti-inflammatory drugs, the decrease in absorption was nullified[30] and it shows that the anti-inflammatory drugs have corrected the decreased absorption capacity of intestine during inflammation. The increased body weight during the treatment of standard drug and TPEE may be due to the restoration of the absorption capacity of the intestine.

CONCLUSION

From the obtained results of the current investigation, it may be concluded that the TPEE at the dose of 300 mg/kg posses significant anti-arthritic activity while TPEAE was less active, also the results of TPEE and cyclophosphamide were a bit similar which reveals that ethanolic extract might posses antiarthritic activity due to presence of sterols. The presence of phytoconstituents such steroids and flavonoids might be responsible the anti-arthritic property. The study needs further extension to identify and characterize the exact active phytoconstituents and to elucidate the exact mechanism of action, which is responsible for the observed significant anti-arthritic activity against adjuvant induced arthritis in rats.

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