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Original Research

Anti-arthritic Activity of *Indigofera tinctoria* L on Adjuvant Induced Arthritis

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Abstract

Indigofera tinctoria L has long been traditionally employed for various diseases, including pain and inflammation. The present study is an attempt to evaluate the potential anti-arthritic activity of *Indigofera tinctoria* L. The antiarthritic activity for petroleum ether and ethanol extracts (100, 200 and 300 mg/kg) of *Indigofera tinctoria* L was carried out by inducing Freunds Complete Adjuvant (FCA) at the tibiotorsal joint. Behavioral changes, vascular permeability, histamine, hematological parameters, ESR, evaluation of development of arthritis, biochemical parameters, and Interleukin (IL) were estimated. *In vitro* proliferation of Spleen cells (MTT assay), Radiographic analysis and histopathological assessments were carried out. The petroleum ether and ethanol extracts of *Indigofera tinctoria* L leaf showed anti-arthritic activity, by significant changes in the behavioral activity, decreased vascular permeability, histamine level, IL and TNF, proliferation of spleenocytes dose-dependently. Similarly the significant increased SOD, GSH and reduced level of lipid peroxidation and decreased ESR, WBC's and lymphocytes were observed. The soft tissue swelling and bone resorption were also reduced. The infiltration of leucocytes and loss of articular cartilage was significantly reduced in groups treated with the extracts. The petroleum ether and ethanol extracts of *Indigofera tinctoria* L showed the potent anti-arthritic activity on FCA induced arthritis in rats.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, autoimmune, systemic inflammatory disease affecting the synovial joints that leads to joint destruction, which is responsible for the deformity and disability (Buch and Emery, 2002). Epidemiology of the arthritis in female: male is 3:1 and prevalence is about 1% of the world population (Katz and Piliero, 1969). The inflammed synovium in RA is characterized by infiltration of leucocytes: predominantly macrophages, T lymphocytes, B cells and dendritic cells. Recent research efforts have been focused on oxidative stress in RA (Cerhan *et al*., 2003; Merry *et al*., 1989). Oxidative injury and inflammatory status in various rheumatic diseases was also confirmed by the increased levels of isoprostanes and prostaglandins in serum and SF (Firestein *et al*., 1997). T lymphocytes derived from the synovial fluid (SF) from RA were known to suffer from severe oxidative stress, with depletion of intracellular glutathione and antioxidant enzyme levels (Maurice *et al*, 1997). The migration of monocytes and lymphocytes infiltration in rheumatoid arthritis synovium is mediated by the abnormal expression of several adhesion molecules (Cunnane *et al*., 2001). Preformed histamine is also considered to be the principal mediator of immediate transient phase of increased vascular permeability, causing venular gaps (Repka-Ramirez and Baraniuk, 2002). TNF and IL are the major cytokines that are produced by activated macrophages, which are responsible for the synthesis of chemokines, growth factors, eicosanoids and nitric oxide (Mantovani *et al*., 1997). Currently, steroids, non-steroidal anti-inflammatory drugs (NSAIDs) and immunosuppressant drugs are used in the relief of inflammation and RA and are often associated with severe adverse effects (Corley *et al*., 2003). This demonstrates the need for new safer drugs. In this regard, natural products have long gained wide acceptance among the public and scientific community.

Indigofera tinctoria L (Fabaceae) commonly known as Indigo is known as medicinal plant since ancient times. The plant has been extensively used in ayurveda and siddha used for Tikita rasam, kata rasam, ushna veeryam, katu, vipaka, anthelmintic, antiperiodic. Roots are used for anti-poison, giddiness, colic, and gonorrhoea. Leaves are used for jaundice, fever, gout (Nandakarni, 2005).

Traditionally, the leaf and roots of the plant are employed for the treatment of anemia, leucorrhoea, inflammation, arthritis and worm infections (Madahava Chetty *et al*, 2008). The plant of *Indigofera tinctoria* L contains glycoside indican, indogotine, indiruben, galactomannan composed of galactose and mannose, alkaloids and flavonoids (Chopra, 1986). It has been pharmacologically validated for its anti-nociceptive (Saravana *et al*, 2009), hepatoprotective (Singh *et al*, 2001), anti-diabetic (Verma *et al*., 2009), antidyslipidemic (Narender *et al*., 2007) activities. The purpose of the study is to know the safe and potent anti-arthritic activity of *Indigofera tinctoria* L on FCA induced arthritis in Sprague-Dawley rats.

MATERIALS AND METHODS

Drugs and Chemicals

Freund's complete adjuvant (FCA), carrageenin, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), 5-5'- dithiobis (2- nitrobenzoic acid), (±) epinephrine, greiss reagent and MTT were purchased from Sigma-Aldrich Co., Spruce Street, St. Louis, MO, USA. Evans blue was purchased from S.D. Fine-Chemicals, Boisar. Histamine, Trypsin *o*-phtalaldehyde, RPMI-1640, Fetal Calf Serum were purchased from Hi-Media, Mumbai. Diclofenac, a gift sample from Novarties India Ltd, Shantistal, Shirgaon, Thane- 401407, India. All reagents, kits and chemicals used in the experiment were of analytical grade.

Plant Material

In the present study, the fresh leafs of the plant *Indigofera tinctoria* L were collected from the Talakona area of Tirumala hills located at Tirupathi, Andhra pradesh, India in the month of July 2009. Herbarium was prepared and authenticated by Dr. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupathi, Andhra pradesh. The leafs are then washed in 10% $KMNO_4$, then dried under shade and pulverized to get moderately coarse powder and passed through sieve (#44). The fine powder was stored in airtight polythene container before extraction. The powder was packed into soxhlet extractor and subjected to successive extraction with petroleum ether (40-60°C) and subsequently with ethanol (60-65°C). After the residue extraction, solvent was distilled off and excess solvent was completely removed by using a rotary flash evaporator to get concentrated, then completely dried in freeze drier (Mini Lyotrap, LTE Scientific Ltd., London) and stored in airtight container in refrigeration until use. The leaf petroleum ether (LPIT) and leaf ethanol extract (LEIT) were used for the antiarthritic activity.

Phytochemical Screening

Phytochemical screening of the crude extract was carried out employing standard procedures and tests (Trease and Evans, 1989), to reveal the presence of chemical constituents such as glycosides, alkaloids, and flavonoids etc.

HPLC Analysis of *Indigofera tinctoria L.*

Chemicals, Reagents and Materials: Methanol of HPLC grade was obtained from Jiangsu Hanbon Sci. & tech. Co., Ltd. (Jiangsu, China). All other chemicals were of analytical reagent grade purchased form Concord Technology Co.

Preparation of Standard Samples: Stock solutions of indirubin (26 µg/ml) was prepared in methanol and stored at 4°C. Stock solution of indirubin was diluted quantitatively with methanol to give working standards. Indirubin calibration standards were prepared at concentrations of 6.5, 32.5, 162, 325, 975 and 1950 ng/ml by spiking 200 µl blank sample extract with indirubin working standard solutions.

Extract Sample Preparation

HPLC analysis was performed using equipment from Shimadazu (Japan): a Shimadazu LC-20AT liquid chromatograph. The mobile phase consisted of a mixture, methanol-water 75:25 V/V) was degassed by suction-filtration through a nylon membrane. The detecting wavelength was 289 nm. The flow rate was 1.0 ml/min. The quantity volume of injecting sample was 6.0 µl. The HPLC system was operated at ambient temperature (28±1ºC).

Acute Toxicity Study

The acute toxicity study was performed as per the method described by Litchfield and Wilcoxon, (1949), and LD_{50} was calculated accordingly. Briefly, the petroleum ether and ethanol extracts of *Indigofera tinctoria* L in the dose range of 10-2000 mg/kg was administered orally to different groups of mice (n=10). The animals were examined at every 30 min up to a period of 3 hr and then, occasionally for additional period of 4 h, finally overnight mortality was recorded. The both extracts had not showed any mortality even at 2000 mg/kg and on this basis of acute toxicity 100, 200 and 300 mg/kg dose were selected for the present study.

Animals

Sprague-Dawley rats of female sex (180-250 g) were obtained from the central animal house of H. S. K. College of Pharmacy and Research Centre, Bagalkot, India. All animals were kept under standard husbandry conditions (21 ± 2^{o}C, relative humidity 55^{o}C ± 10 ^{o}C) for 12hr dark and 12hr light cycle respectively in standard propylene cages. The animals were fed with standard food (Amruth, Sangli, Maharastra) and water *ad libitum*. All the experiments were conducted in accordance with direction of Institutional Animals Ethics Committee (HSKCP/IAEC, Clear / 2008-09 / 1-8) and CPCSEA guide lines.

Anti-inflammatory Activity by Carrageenin Induced Paw Edema

The anti-inflammatory activity of Leaf petroleum extract of *Indigofera tinctoria* L (LPIT) and Leaf ethanol extract of *Indigofera tinctoria* L (LEIT) was carried out as described by Araico *et al*, (2007) and overnight-starved Sprague-Dawley rats were divided into 9 groups, each group containing 6 animals.

Group I: Normal group receives the vehicle (5% Tween 80)

Group II: Control group receives vehicle (5% Tween 80) + Carrageenin induced changes in rat paw edema

Group III: Effect of Standard (Diclofenac 10 mg/kg) on Carrageenin induced changes in rat paw edema

Group IV: Effect of LPIT (100 mg/kg) on Carrageenin induced changes in rat paw edema

Group V: Effect of LPIT (200 mg/kg) on Carrageenin induced changes in rat paw edema

Group VI: Effect of LPIT (300 mg/kg) on Carrageenin induced changes in rat paw edema

Group VII: Effect of LEIT (100 mg/kg) on Carrageenin induced changes in rat paw edema
Group VIII: Effect of LEIT (200 mg/kg) on Carrageenin induced changes in rat paw edema
Group IX: Effect of LEIT (300 mg/kg) on Carrageenin induced changes in rat paw edema

The one hour after the administration of all the above treatments, in different groups, rats in all groups challenged Carrageenan (1% prepared in 0.4% NaCl) in sub-plantar region of right hind paw. The paw volume was measured at different intervals of level 0, 0.5, 1, 2, 3, 4 and 5 hr using digital Plethysmometer (UGO Basil, Italy) after the administration of Carrageenan.

Percentage inhibition (%) = [1- volume in ml (Test extract) / Volume in ml (Control)] × 100.

Anti-arthritic Activity by FCA Induced Arthritis in Rats

The anti-arthritic activity of Leaf petroleum extract of *Indigofera tinctoria* L (LPIT) and Leaf ethanol extract of *Indigofera tinctoria* L (LEIT) was carried out as described by Monica *et al*. (2004) and female Sprague-Dawley rats were divided into 9 groups, each group containing 6 animals, for acute and chronic models separately.

Group I: Normal group receives the vehicle (5% Tween 80)
Group II: Control group receives vehicle (5% Tween 80) + FCA induced changes in rat paw edema
Group III: Effect of Standard group (Diclofenac 10 mg/kg) on FCA induced changes in rat paw edema
Group IV: Effect of LPIT (100 mg/kg) on FCA induced changes in rat paw edema
Group V: Effect of LPIT (200 mg/kg) on FCA induced changes in rat paw edema
Group VI: Effect of LPIT (300 mg/kg) on FCA induced changes in rat paw edema
Group VII: Effect of LEIT (100 mg/kg) on FCA induced changes in rat paw edema
Group VIII: Effect of LEIT (200 mg/kg) on FCA induced changes in rat paw edema
Group IX: Effect of LEIT (300 mg/kg) on FCA induced changes in rat paw edema

Acute Antiarthritic Activity

All the animals were anesthetized (45 mg/kg of Ketamine chloride), and 0.1ml of Freund's complete adjuvant was injected into the tibiotorsal joint of the rats. The animals were carefully and thoroughly inspected everyday by examining the affected paw and the animals general status. In control, the tibiotorsal injection of FCA produces local edema after few hours with a progressive increase reaching its maximum effects by 8^{th} day after administration. On days 0, 5, 6, 7 and 8^{th} the behavioral parameters were examined and paw volume are measured in all groups (Monica *et al*., 2004).

Chronic Antiarthritic Activity

Following anesthesia (45 mg/kg of Ketamine chloride), 0.1ml of Freund's complete adjuvant was injected into the tibiotorsal joint of the rats. Everyday animals were carefully and thoroughly inspected, by examining the affected paw and the animals general status. In control animals, the tibiotorsal injection of FCA produces local edema after few hours with a progressive increase reaching its maximum in 40^{th} day after administration. On 0, 10, 20, 24, 29, 34 and 40^{th} days, the behavioral parameters were examined and paw volume is measured thoroughly in all groups (Monica *et al*., 2004).

Evaluation of Behavioral Parameters in Acute and Chronic Arthritis on Open Field Test

The effect of the plant extracts on behavioral parameters in acute and chronic models were carried out in which rat was placed in an open field in the sound-attenuated room. The floor has white polyvinyl with a black grid dividing open field into 84 squares (10×10). Illumination was provided by a bulb (60 W) placed above the center of the field, while the rest of the room was darkened. The rat was initially placed in the center of field and observed for 5 minutes in all tests, latency time to start explore the open field (sec), horizontal locomotor activity (grid line crossed) (Dimitrijevic *et al*., 2001), rearing (rat looks for some thing in the air by elevating its head and forepaws) (Costa *et al*., 1981), grooming (rubbing the nose with its forepaws and preening), intense of defecation (number of boluses), were recorded. In between trials the box was cleaned with wet sponge and tissue paper. All the observations were made between 18.00 and 20.00 h (Vane *et al*., 1987).

Measurement of Paw Edema Volume in Acute and Chronic Arthritis

The inflammation in the paw edema volume during acute and chronic arthritis was measured by using Digital Plethysmograph (7141, UGO BASILE). The change in the paw volume for the extracts of LPIT and LEIT were estimated.

Percentage inhibition (%) = [1- volume in ml (Test extract) / Volume in ml (Control)] × 100.

Assessment of Vascular Permeability in Chronic Arthritis

The effect of *Indigofera tinctoria* extracts for the assessment of vascular permeability in chronic arthritis was carried out as described by Franchis *et al*, (2004). Evan's blue 50 mg/kg of body weight was administered via the jugular vein into the anaesthetized rat. After 4hr of Evans blue administration, the anterior and posterior synovial capsules and fat pad were dissected from each knee joint. The tissues obtained from each knee were then weighed, and the amount of evans blue in the sample was estimated by extracting the dye by following procedure. This entailed cutting the capsule in to smaller pieces and mixing them with acetone in 1% $NaSO_4$ in the ratio of 7:3. The samples were shaken gently and continuously for 24 h at room temperature. Each preparation was centrifuged for 10 min at 2000 rpm and 2 ml of the supernatant was separated for measurement of absorbance at 620 nm using UV- spectrophotometer (UV-1601, Shimadzu Corporation, Kyoto, Japan). The amount of dye recovered content of vascular permeability was calculated by extrapolating with standard curve prepared with different concentrations of evans blue solution.

Evaluation of Development of Arthritis in Chronic Arthritis

The effect of extracts on evaluation of development of arthritis on FCA induced arthritis was carried out as described by Tomita *et al*. (2006). Evaluation is assessed for every five days in 21^{st}, 26^{th}, 31^{st}, 36^{th} and 40^{th} days after the administration of FCA. It has been observed by two blinded observers using a three point scale for each paw; 0 =normal joint, 1 =slight inflammation and redness; 2 =severe erythema and swelling affecting the entire paw; with inhibition of use; and 3 =deformed paw or joint with ankylosis, joint rigidity, joint rigidity, and loss of function.

Serum Biochemical Parameters in Chronic Arthritis

At the end of the treatment, blood was collected from each group of the animal by retro-orbital puncture and centrifuged for 3000 rpm for 10 min to separate serum for the estimation of Creatinine (Murray *et al*., 1984), ALP (Moss *et al*., 1994), AST (Burtis *et al*., 1986), ALT (Bradely *et al*., 1972) and Nitric oxide (Annie Shirwaikar *et al*., 2003).

Antioxidant Enzyme Estimation in Tissues for Chronic Arthritis

The animals were sacrificed after the end of the treatment by decapitation. The liver was removed and washed in cooled 0.9 % saline, kept on ice and subsequently blotted on filter paper, then weighed and homogenized in cold phosphate buffer (0.1 M, pH 7.4). The homogenates were centrifuged at 10000 rpm for 10 min at 4 ºC (MPW-350R, Korea) and post-mitochondrial supernatant (PMS) was used for the estimation of Lipid peroxidation (Braughler *et al*., 1987). The supernatant was again centrifuged at 17000 rpm for 1 hr at 4ºC. The mitochondrial supernatant obtained was used for further estimation of SOD (Misra and Fridovich, 1972), CAT Claiborne (1985) and GSH (Chandrashekhar *et al*., 2010).

Determination of Release of Histamine from Blood and Liver in Chronic Arthritis

The oxalated blood (1 ml) was shaken thoroughly and centrifuged at 400 rpm for 10 min. the hemolyzed blood treated with 4.5 ml of distilled water and 0.5 ml of 12 % concentrated perchloric acid, then allowed to stand for 10 min and centrifuged (Parkhurst *et al*., 2010). The supernatant was used for measuring the histamine content by *o*-pthaldialdehyde spectrofluorimetric method. The fluorescence intensity was measured at emission 438nm and excitation 353 nm using spectrofluorimeter (Shore *et al*., 1959).

Hematological Parameters and E.S.R Estimation in Chronic Arthritis

The effect of the plant extracts for the estimation of hematological parameters was carried out and estimated according to Shankarnarayan *et al*, (2009).Blood samples were subjected to hemocytometer (Symex KX-21) for the determination of White Blood Cells (W.B.C), Red Blood Cells (R.B.C), Hemoglobin (Hb), Mean Cell Hemoglobin (MCH), Mean Cell Hemoglobin Concentration (MCHC), Mean Cell Volume (MCV), Platelets, Hematocrit (HCT), Lymphocytes The determination of E.S.R was carried out and estimated according to Jain *et al*, (2000).

Estimation of Serum and Tissue Levels of IL

The levels of ILs were measured by using commercial available Enzyme Linked Immunosorbent Assay (ELISA) kit (Pearson and Wood, 1963).

Radiographic Analysis and Histopathalogical Assessment in Chronic Arthritis

The radiographic analysis was carried out according to Kalpesh *et al*, (2009). On day 40, animals were anesthetized with ketamine (45mg/kg). Radiographs of the FCA injected joints were taken with a Dental X-ray machine. Rats were placed on a radiographic box at a distance of 90 cm from the X-ray source. The X-ray image of the FCA injected joints of each rat was evaluated for radiographic changes.

All the animals were sacrificed at the end of the experiment. Left hind paws of one animal from each group was removed and fixed in 10% formalin for 24 h. Metatarsophalangeal joints were sampled and blocked in paraffin wax after processing the tissues in alcohol for 16 h. 4 µm thick sections from each block were stained with haematoxylin and eosin (Chakraborty *et al*, 2008).

***In vitro* Proliferation of Spleen Cells (MTT) Assay in Chronic Arthritis**

In vitro proliferation of spleen cells was carried out and estimated according to Tomita *et al*, (2006) using by 3-[-4, 5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Spleens were removed on 40th day. Red blood cells were removed by treatment with 0.16 Tris NH_4Cl solution and cell suspensions (5 × 10^6 cells/well in a flat bottom, 96 well plate) were cultured along with 5% mixture of gentamycin (3µg/ml), penicillin (100 units/ml) for 72 hr at 37°C in 5% CO_2 in RPMI medium containing 50µg/ml heat-denatured bovine CII (heated for 10 min at 80°C) or 1µg/ml phytohemagglutinin (PHA) and routinely subculture with 0.25% trypsin – 0.02% EDTA solution. On the day of assay, MTT (0.5mg/ml) was added to the medium in each well, and plates were returned to incubator for 1hr. Plates were centrifuged (500 × g for 10 min). Supernatants were removed, and 100µl of dimethyl sulphoxide (DMSO) were added. The plates were agitated in the dark for 10 min to dissolve the MTT formazan crystals. Then the percentage inhibition of proliferation of spleen cells was carried out using the formula.

Percentage of inhibition= [control absorbance-test absorbance/control absorbance]x 100

Statistical Analysis

All the data are presented as mean ± SEM. The significance of difference in means between control and treated animals for different parameters was determined by using One-way Analysis of Variance (ANOVA) followed by multiple comparisons Dunnett's test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Anti-inflammatory Activity of Carrageenan Induced Paw Edema

The control group animals had shown the significant increase ($p< 0.05$ to $p<0.001$) in the paw edema. The *Indigofera tinctoria* L extract treated groups had shown the significant decrease ($p<0.05$, to $p<0.001$) in the paw edema and percentage inhibition (9.07 to 33), this indicates the potent anti-inflammatory activity (Table1).

Behavioral Parameters

In the present study, the behavioral parameters are assessed in *Indigofera tinctoria* L extracts treated animals against FCA induced acute and chronic arthritis in rats. The anti-arthritic activity of *Indigofera tinctoria* L showed significant activity against FCA induced acute arthritis and chronic arthritis models. There is significant decrease in the ambulatory movement ($p<0.05$ to $p<0.001$), rearing ($p<0.05$, to $p<0.001$), paw volume ($p<0.05$ to $p<0.001$) and increase in the grooming behavior ($p<0.05$ to $p<0.001$) in control group as compared to normal group in acute and chronic arthritis. The standard drug Diclofenac, petroleum and ethanol extracts of *Indigofera tinctoria* L has significantly increased the ambulatory movement ($p<0.05$ to $p<0.001$), rearing ($p<0.05$ to $p<0.001$), latency ($p<0.05$ *to* $p<0.001$) and significantly decreased the grooming behavior ($p<0.05$ to $p<0.001$) in acute (Table 2 to 5) and chronic (Table 6 to 9) treatments in dose dependently respectively.

Table 1: Effect of petroleum and ethanol extracts of *Indigofera tinctoria* L on Carrageenin induced rat paw edema

Treatment groups	Paw volume in ml (Percentage of inhibition)						
	0 hr	0.5 hr	1 hr	2 hr	3 hr	4 hr	5 hr
Normal	1.05±0.02	0.99 ± 0.09	1 .0 ± 0.02	1.06 ± 0.03	1.07 ± 0.01	1.08 ± 0.01	1.03 ± 0.02
Control	1.20 ± 0.02*	1.45 ± 0.06**	1.87 ± 0.02***	2.2 2± 0.05***	2.37 ± 0.05***	2.14 ± 0.02***	2.06 ± 0.02***
Diclofenac (10 mg/kg)	1.20 ± 0.06	1.26 ± 0.03b (13.1)	1.26 ± 0.03c (32.5)	1.25 ± 0.02c (43.5)	1.22 ± 0.01c (48.5)	1.27 ± 0.02c (40.6)	1.26 ± 0.008c (38.9)
LPIT (100 mg/kg)	1.20 ± 0.03	1.25 ± 0.02b (14.0)	1.84 ± 0.01 (1.70)	2.04 ± 0.03a (8.09)	2.01 ± 0.02b (15.7)	1.93 ± 0.01b (9.77)	1.86 ± 0.01c (9.78)
LPIT (200 mg/kg)	1.08 ± 0.04	1.22 ± 0.01c (15.6)	1.85 ± 0.01 (0.91)	1.78 ± 0.02c (19.6)	1.74 ± 0.01c (26.0)	1.7 ± 0.008c (20.8)	1.67 ± 0.01c (18.8)
LPIT (300 mg/kg)	1.07 ± 0.05	1.17 ± 0.02c (19.3)	1.70 ± 0.04a (9.07)	1.65 ± 0.03c (25.4)	1.59 ± 0.01c (33.0)	1.55 ± 0.01c (27.7)	1.43 ± 0.02c (30.5)
LEIT (100 mg/kg)	1.10 ± 0.04	1.40 ± 0.06 (3.43)	1.87 ± 0.12 (0.00)	2.08 ± 0.07 (6.47)	2.27 ± 0.08 (4.30)	2.20 ± 0.06 (0.00)	2.04 ± 0.05 (0.00)
LEIT (200 mg/kg)	1.17 ± 0.02	1.45 ± 0.03 (0.00)	1.86 ± 0.04 (0.00)	1.87 ± 0.02c (15.9)	1.91 ± 0.02c (19.4)	1.86 ± 0.02c (13.0)	1.81 ± 0.02c (9.2)
LEIT (300 mg/kg)	1.09 ± 0.03	1.45 ± 0.05 (0.00)	1.76 ± 0.06 (5.76)	1.86 ± 0.02c (16.0)	1.80 ± 0.01c (24.0)	1.76 ± 0.01c (17.6)	1.70 ± 0.01c (17.5)

Values are expressed in mean ± SEM, n=6. One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to normal group and $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ as compared with control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria* L; LEIT = Leaf ethanol extract of *Indigofera tinctoria* L.

Table 2: Effect of petroleum and ethanol extracts of *Indigofera tinctoria* L on FCA induced acute arthritis on rat ambulatory movement

Treatment groups	0th Day	5th Day	6th Day	7th Day	8th Day
Normal (5%tween80)	84.33±4.876	89.00±10.02	95.67±2.186	83.83±2.822	79.17±7.346
Control (5%tween80)	90.66±3.125[a]	65.17±2.182[a]	73.00±3.235[a]	48.00±5.335[a]	45.67±3.293[a]
Diclofenac (10 mg/kg)	44.33±2.499*	58.00±1.732	76.33±1.687	96.8±121.0*	84.83±1.600**
LPIT (100 mg/kg)	61.50±5.470	72.50±6.587	92.33±6.114	93.67±5.897	71.50±3.481
LPIT (200 mg/kg)	59.50±4.137*	65.33±2.906	71.50±3.452	71.00±3.454	76.50±2.814*
LPIT (300 mg/kg)	57.00±3.847*	59.83±4.028	65.83±5.069	68.17±3.439	97.67±7.324***
LEIT (100 mg/kg)	87.33±5.846	102.8±3.995***	90.67±7.424	84.17±15.74	96.17±17.91***
LEIT (200 mg/kg)	66.50±2.566	102.7±4.638***	86.67±8.297	101.7±8.341	92.83±4.854***
LEIT (300 mg/kg)	59.83±9.569	123.5±6.469***	98.17±5.986*	114.8±3.628	117.5±5.271***

All values are expressed as mean ± SEM, n=6, One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant. $^{a}p<0.001$ as compared to normal group and *$p<0.05$, **$p<0.01$, ***$p< 0.001$ as compared to control group; LPIT = Leaf petroleum ether extract of *Indigofera tinctoria* L; LEIT = Leaf ethanol extract of *Indigofera tinctoria* L.

Table 3: Effect of petroleum and ethanol extracts of *Indigofera tinctoria* L on FCA induced acute arthritis on rat rearing

Treatment groups	0th Day	5th Day	6th Day	7th Day	8th Day
Normal (5%tween80)	24.17±3.070	19.33±3.293	19.33±3.293	19.33±3.293	19.33±3.293
Control (5%tween80)	10.00±1.183[a]	10.50±1.668[a]	17.67±1.944[a]	17.67±1.944[a]	9.667±1.202[a]
Diclofenac (10 mg/kg)	12.83±0.8333	20.00±1.155***	20.17±1.167	20.17±1.167	22.00±1.461***
LPIT (100 mg/kg)	11.33±1.453	14.50±1.607	16.67±1.145	16.67±1.145	18.67±0.6667*
LPIT (200 mg/kg)	4.167±1.108	16.83±1.302*	20.50±1.408	20.50±1.408	20.83±1.621**
LPIT (300 mg/kg)	7.167±1.537	17.67±1.476**	22.17±1.400	22.17±1.400	20.67±0.8433**
LEIT (100 mg/kg)	3.833±1.759	14.67±1.978	12.33±1.116	12.33±1.116	16.33±2.716
LEIT (200 mg/kg)	11.00±1.291	21.67±1.229***	12.17±1.249	12.17±1.249	20.67±2.679**
LEIT (300 mg/kg)	11.00±0.7746	22.17±1.537***	18.33±2.044	18.33±2.044	22.00±2.206***

All values are expressed as mean ± SEM, n=6, One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant. $^{a}p<0.001$ as compared to normal group and *$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria*; LEIT = Leaf ethanol extract of *Indigofera tinctoria* L.

Table 4: Effect of petroleum and ethanol extracts of *Indigofera tinctoria* L on FCA induced acute arthritis on rat grooming

Treatment groups	0th Day	5th Day	6th Day	7th Day	8th Day
Normal (5%tween80)	7.833±0.8724	7.833±0.8724	7.500±0.9220	12.00±0.8563	11.83±2.386
Control (5%tween80)	8.333±8.333[a]	8.333±1.606[a]	14.17±0.9098[a]	11.00±1.528[a]	11.50±0.9916[a]
Diclofenac (10 mg/kg)	7.500±1.648	4.833±0.6009	3.000±0.7303***	3.167±0.8333**	3.167±0.7491***
LPIT (100 mg/kg)	10.33±0.9888	10.00±1.183	11.50±1.979	14.67±1.838	15.00±1.751
LPIT (200 mg/kg)	11.67±1.687	11.17±2.056	8.500±1.147	9.167±1.302	11.17±1.249
LPIT (300 mg/kg)	12.67±1.453	12.33±1.606	8.500±2.349	9.000±1.713	5.667±0.557**
LEIT (100 mg/kg)	10.83±1.641	10.00±2.000	12.33±1.116	6.333±1.202	5.000±0.8563**
LEIT (200 mg/kg)	11.50±0.9574	10.83±1.108	10.33±1.978	6.000±1.291	7.333±1.202
LEIT (300 mg/kg)	8.000±0.8165	6.833±1.493	7.167±0.8724*	7.66±0.3333	9.167±1.537

All values are expressed as mean ± SEM, n=6, One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant. $^{a}p<0.001$ as compared to normal group. *$p< 0.05$,**$p<0.01$,***$p<0.001$ as compared to control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria* L; LEIT = Leaf ethanol extract of *Indigofera tinctoria* L.

Table 5: Effect of petroleum and ethanol extracts of *Indigofera tinctoria* L on FCA induced acute arthritis on rat paw edema

Treatment groups	0th Day	5th Day	6th Day	7th Day	8th Day
Normal(5%tween80)	0.9600±0.0242	1.008±0.0254	0.9883±0.0153	0.9817±0.0157	0.9883±0.0246
Control(5%tween80)	1.620±0.056[a]	2.420±0.0383[a]	2.440±0.0413[a]	2.523±0.0359[a]	2.523±0.0359[a]
Diclofenac (10 mg/kg)	1.340±0.035**	2.387±0.0241	2.242±0.0190	2.128±0.0166***	2.083±0.0662***
LPIT (100 mg/kg)	1.795±0.0441	2.468±0.0496	2.528±0.0675	2.462±0.0456	2.263±0.0322*
LPIT (200 mg/kg)	1.865±0.0399	2.437±0.0320	2.438±0.0416	2.197±0.1266**	2.348±0.0611***
LPIT (300 mg/kg)	1.800±0.0359	2.457±0.0378	2.187±0.0588*	2.027±0.0398***	2.137±0.0589***
LEIT (100 mg/kg)	1.602±0.0989	2.290±0.0505	2.548±0.0373	2.568±0.0414	2.475±0.0450
LEIT (200 mg/kg)	1.570±0.0614	2.415±0.102	2.350±0.119	2.398±0.0691	2.092±0.0801***
LEIT (300 mg/kg)	1.737±0.0548	2.218±0.0252*	2.065±0.0416***	2.167±0.0609***	1.992±0.0501***

All values are expressed as mean ± SEM, n=6, One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant. [a]$p<0.001$ as compared to normal group and *$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria* L; LEIT = Leaf ethanol extract of *Indigofera tinctoria* L.

Table 6: Effect of petroleum and ethanol extracts of *Indigofera tinctoria* L on FCA induced chronic arthritis on rat ambulatory movement

Treatment groups	0th Day	10th day	20th day	24th day	29th day	34th day	40th day
Normal (5% tween80)	103.8±5.45	101.2±3.390	104.7±5.45	106.8± 3.76	115.2±6.940	115.3± 4.64	128.8±12.02
Control (5% tween80)	49.33±3.99	67.17±4.061[a]	69.00±4.64[a]	46.83± 9.08[a]	54.83±7.296[a]	57.50±7.531[a]	54.50± 6.970[a]
Diclofenac (10 mg/kg)	47.00±1.770	75.17±3.572	83.83±2.971	87.00±2.20***	95.5±1.70***	104.3±2.24***	109.8±2.469***
LPIT (100 mg/kg)	61.17±2.626*	75.6± 4.50***	76.33±14.03	100.7± 11.6***	100.2±8.39***	102.7±8.93***	108.7±7.740***
LPIT (200 mg/kg)	61.67±2.34**	66.67±2.848*	96.17±10.89*	93.17±5.764***	96.50±4.12***	99.67±3.97***	102.7±3.913***
LPIT (300 mg/kg)	62.33±4.34**	69.33±4.492*	124.2±7.23***	132.3±6.339***	135.8±5.60***	135.8±5.6***	136.0±5.675***
LEIT (100 mg/kg)	65.0± 1.91***	99.00±12.29	76.33±4.77	77.83±5.885**	81.17±7.79**	81.67±8.16*	80.83±8.002**
LEIT (200 mg/kg)	64.83± 1.9***	90.83±4.820	70.17±2.34	70.17±2.040	69.83±1.81	71.83±1.138	73.17±1.046
LEIT (300 mg/kg)	68.67± 3.0***	44.50±3.041	69.0±6.909	70.0±6.952	72.17±7.045	72.83±5.741	77.50±4.610*

All values are expressed as mean ± SEM, n=6, One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant; [a]$p<0.001$as compared to normal group and *$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria*; LEIT = Leaf ethanol extract of *Indigofera tinctoria*

Table 7: Effect of petroleum and ethanol extracts of *Indigofera tinctoria* L on FCA induced chronic arthritis on rat rearing

Treatment groups	0th Day	10th day	20th day	24th day	34th day	40th day
Normal (5% tween80)	27.67±2.616	28.17±3.381	31.67± 2.060	32.00±1.390	21.17±1.327	21.17±1.327
Control (5% tween80)	9.167±1.53[a]	12.67±1.56[a]	15.83± 1.53[a]	15.83±1.537[a]	16.00±1.291[a]	17.50±0.8466[a]
Diclofenac (10 mg/kg)	13.00±0.856	16.67±1.764	21.50±1.204	23.33±0.881	30.00±1.15***	33.33±1.45***
LPIT (100 mg/kg)	3.000±0.730	13.50±1.522	20.00± 3.890	21.67±1.647	19.00±2.556	21.50±1.586
LPIT (200 mg/kg)	4.000±1.065	19.67±2.985*	23.17± 5.425	27.17±1.014	25.17±1.83***	25.67±3.44***
LPIT (300 mg/kg)	8.000±1.155	24.00±2.38***	26.00± 1.36*	28.17±0.909	33.00±1.31***	33.00±1.31***
LEIT (100 mg/kg)	6.000±1.342	6.333±1.174*	8.333±0.760	11.67±0.918	16.83±1.400	19.67±1.333
LEIT (200 mg/kg)	8.500±2.405	9.000±0.9661	11.00±0.856	12.00±0.730	18.00±1.000	19.83±0.6540
LEIT (300 mg/kg)	7.667±0.988	14.67±0.9545	20.67±1.256	19.83±0.654	20.83±0.8333	20.83±0.9804

All values are expressed as mean ± SEM, n=6, One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant; [a]$p<0.001$as compared to normal group and *$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria*; LEIT = Leaf ethanol extract of *Indigofera tinctoria*.

Table 8: Effect of petroleum and ethanol extracts of *Indigofera tinctoria* L on FCA induced chronic arthritis on rat grooming

Treatment groups	0th Day	10th day	20th day	24th day	29th day	34th day	40th day
Normal (5% tween 80)	10.50±0.9916	11.00±0.6831	11.00±0.7303	13.00±1.528	7.500±0.9916	8.000±0.5774	6.000±0.5774
Control (5% tween 80)	7.000 ±2.33[a]	10.83± 0.7032[a]	12.00 ± 1.612[a]	12.67 ± 1.498[a]	13.33 ±1.202[a]	14.67±1.542[a]	12.67±0.802[a]
Diclofenac (10 mg/kg)	9.500±0.9220	5.500±0.8466*	2.667±0.614***	2.500±0.223***	4.333±3.283***	1.000±0.4472	1.833±0.47***
LPIT (100 mg/kg)	2.500±0.6708	9.167±1.138	8.167±1.302*	8.167±1.579*	7.833±1.470*	7.167±0.98***	7.833±1.07***
LPIT (200 mg/kg)	3.333±0.6146	8.333±1.202	9.500±0.9916	10.67±0.8819	11.83±1.376	9.833±1.833**	9.667±0.91*
LPIT (300 mg/kg)	8.167±1.352*	8.333±1.874	10.50±1.088	10.83±1.537	8.667±0.8433	9.833±1.10**	9.833±1.108
LEIT (100 mg/kg)	3.167±0.6009	6.167±0.6009	6.167±0.477***	8.167±0.703*	4.667±0.843***	4.833±0.83***	2.667±0.76***
LEIT (200 mg/kg)	4.833±0.9098	4.833±0.9098**	7.667±0.6146*	6.667±0.666***	4.500±0.500***	5.667±0.80***	2.667±0.66***
LEIT (300 mg/kg)	13.67±0.8433	8.667±2.108	11.67±0.6146	10.00±0.7303	8.667±0.666	8.667±0.42***	6.000±0.73***

All values are expressed as mean ± SEM, n=6, One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant; [a]$p<0.001$as compared to normal group and * $p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria*; LEIT = Leaf ethanol extract of *Indigofera tinctoria*

Table 9: Effect of petroleum and ethanol extracts of *Indigofera tinctoria* L on FCA induced chronic arthritis on rat paw edema

Treatment groups	0 day	10th day	20th day	24th day	29th day	34th day	40th day
Normal (5%tween80)	1.07 ± 0.028	1.102 ± 0.016	1.10 ± 0.024	1.085 ± 0.0192	1.148 ± 0.0252	1.14 ± 0.029	1.14 ± 0.022
Control (5%tween80)	1.43 ± 0.041[a]	2.59 ± 0.101[a]	2.91 ± 0.037[a]	2.960 ± 0.0371[a]	3.330± 0.11[a]	3.37 ± 0.084[a]	3.46 ± 0.103[a]
Diclofenac (10 mg/kg)	1.35 ± 0.021	2.20 ± 0.02***	2.06 ± 0.03***	1.94 ± 0.027***	1.878± 0.02***	1.80 ± 0.03***	1.72± 0.029***
LPIT (100 mg/kg)	1.41 ± 0.042	2.588 ± 0.079	2.37 ± 0.04***	2.34 ± 0.091***	2.20±0.098***	2.15± 0.066***	2.063± 0.05***
LPIT (200 mg/kg)	1.45 ± 0.031	2.582 ± 0.068	2.30 ± 0.02***	2.25 ± 0.02***	2.193± 0.02***	2.12± 0.028***	2.05± 0.029***
LPIT (300 mg/kg)	1.43 ± 0.041	2.568 ± 0.052	2.11 ± 0.09***	2.09 ± 0.058***	1.97 ± 0.038***	1.86± 0.030***	1.81± 0.037***
LEIT (100 mg/kg)	1.49 ± 0.188	2.587 ± 0.028	2.76 ± 0.026	2.70 ± 0.024**	2.63 ± 0.023***	2.56± 0.02***	2.46± 0.061***
LEIT (200 mg/kg)	1.39 ± 0.029	2.580 ± 0.087	2.61 ± 0.60**	2.63 ± 0.061***	2.55 ± 0.064***	2.477± 0.07***	2.39± 0.055***
LEIT (300 mg/kg)	1.34 ± 0.019	2.560 ± 0.101	2.60 ± 0.079**	2.58 ± 0.072***	2.53 ± 0.062***	2.500± 0.06***	2.33± 0.030***

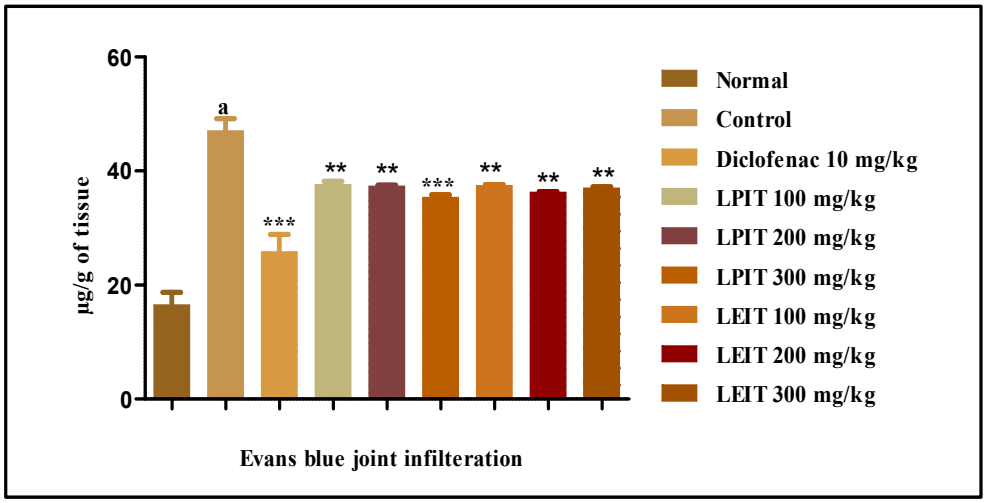
All values are expressed as mean ± SEM, n=6, One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant; [a]$p<0.001$as compared to normal group; *$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria*; LEIT = Leaf ethanol extract of *Indigofera tinctoria*

Assessment of Vascular Permeability

The results as indicated in figure 1, showed significant increase in the Evans blue concentration was seen in the control group ($p<0.001$) as compared to the normal group. The petroleum ether and ethanol extracts of *Indigofera tinctoria* L has showed significant ($p<0.01$, $p<0.001$) decrease in the Evans blue concentration dose dependently.

Evaluation of Development of Arthritis

The results of development of arthritis is presented in Table 10. The inflammatory score in the control group showed significantly increased ($p<0.001$) as compared to normal group. The extracts treated group animals showed a significant decrease ($p<0.05$ to $p<0.001$) in redness, swelling, and erythema at ankle joints as compared to control group.



All values are expressed as mean ± SEM, n=6, One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of p<0.05 was considered as significant; [a]p<0.001 as compared to normal group and *p<0.05, **p<0.01, ***p<0.001 as compared to control group. LPIT = Leaf petroleum ether extract of Indigofera tinctoria L; LEIT = Leaf ethanol extract of Indigofera tinctoria L.

Figure 1: Effect of the petroleum and ethanol extracts of Indigofera tinctoria L on FCA induced Evans blue joint infiltration (Vascular permeability).

Table 10: Effect of the petroleum ether and ethanol extracts of *Indigofera tinctoria* L on evaluation of development of arthritis in FCA induced rat

Day	Normal	Control	Diclofenac (10 mg/kg)	LPIT (100mg/kg)	LPIT2 (200mg/kg)	LPIT3 (300mg/kg)	LEIT (100mg/kg)	LEIT4 (200mg/kg)	LEIT5 (300mg/kg)
21st	0.0±0.0	2.1±0.21***	1.75±0.11	2.00±0.00	1.83±0.30	1.83 ± 0.10	2.33 ± 0.21	2.00 ± 0.00	2.00 ± 0.0
26th	0.0±0.0	2.2±0.17***	1.41±0.15a	1.58 ± 0.23	1.66 ± 0.33	1.66 ± 0.16	2.25 ± 0.17	2.00 ± 0.00	1.91 ± 0.08
31st	0.0±0.0	2.41±0.20***	1.16±0.16[c]	1.41±0.20[b]	1.50±0.22[b]	1.33± 0.16[c]	2.25 ± 0.16	2.00 ± 0.00	1.75 ± 0.17
36th	0.0± 0.0	2.58±0.20***	1.08± 0.08[c]	1.41± 0.20[c]	1.33± 0.21[c]	1.00± 0.00[c]	2.00 ±0.10	1.83±0.16[a]	1.33±0.21[b]
40th	0.0±0.0	2.66±0.21***	1.00 ± 0.0[c]	1.33±0.21[c]	1.33± 0.20[c]	0.83± 0.16[c]	2.00 ± 0.00	1.66± 0.21[c]	1.30± 0.21[c]

Values are expressed in mean ± SEM, n=6. One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant. ***$p<0.001$ as compared to normal group and [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ as compared with control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria* L; LEIT = Leaf ethanol extract of *Indigofera tinctoria* L

Serum Biochemical Parameters

In the present study, the levels of ALP (p<0.001), AST (p<0.001), ALT (p<0.001), creatinine (p<0.001) and nitric oxide (p<0.01, p<0.001) were significantly increased in the arthritis control group as compared to normal group. The petroleum ether and ethanol extracts of *Indigofera tinctoria* L had shown the significant decrease in ALP (p<0.001), AST (p<0.05 to p<0.001), ALT (p<0.01, p<0.001), creatinine (p<0.001) and nitric oxide (p<0.001) as compared to control group (Table 11).

Table 11: Effect of the Serum biochemical estimation of petroleum ether and ethanol extracts of *Indigofera tinctoria* L on FCA induced rat chronic arthritis

Parameters	Normal	Control	Diclofenac (10 mg/kg)	LPIT (100mg/kg)	LPIT2 (200mg/kg)	LPIT3 (300mg/kg)	LEIT (100mg/kg)	LEIT4 (200mg/kg)	LEIT5 (300mg/kg)
ALP (IU/L)	197.7±10.7	631.9±57.3***	219.3±9.06^c	268.9±14.6^c	270.5 ± 7.2^c	214.8±9.7^c	588.4 ± 16.8	507.6 ± 6.54^c	425.5 ± 20.6^c
AST (IU/L)	23.36±6.27	428.3±61.4***	27.3±5.16^c	311.9±26.7	220.6±39.8^c	49.1±17.3^c	322.5 ± 70.9	256.3 ± 25.4^a	182.6 ± 57.5^c
ALT (IU/L)	1483±51.86	2492 ±17.5***	1628±18.9	2016±51.2^c	1858±19.63^c	1458±11.1^c	2279 ± 37.4^c	2261 ± 101.4	2012 ± 15.5^b
Creatinine (mg/dL)	0.69±0.07	1.96 ± 0.03***	0.57±0.06^c	0.92±0.11^c	0.88±0.05^c	0.72±0.02^c	1.87 ± 0.03	1.49 ± 0.03^c	1.31 ± 0.01^c
Nitric oxide (n mole/mg of serum)	0.049±0.0	0.092±0.04***	0.035±0.02^c	0.082±0.05	0.076±0.02	0.066±0.09^c	0.088 ± 0.01	0.083 ± 0.08	0.078 ± 0.01

Values are expressed in mean ± SEM, n=6. One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of p<0.05 was considered as significant. ***p<0.001 as compared to normal group and $^a p$<0.05, $^b p$<0.01, $^c p$<0.001 as compared with control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria* L; LEIT = Leaf ethanol extract of *Indigofera tinctoria* L.

Antioxidant Estimation in Tissues

The results are shown in Table 12 revels the potential anti-arthritic activity of *Indigofera tinctoria* L. LPO levels (p<0.001) exhibited significant elevated and enzymatic and non-enzymatic parameters SOD (p<0.001), CAT (p<0.001) and GSH (p<0.001) showed a significant decrease in the FCA induced arthritis group. The animals from extracts treated groups had shown a significant protection by reducing the elevated levels of LPO (p<0.001) and marked increase in SOD (p<0.05, p<0.01), CAT, GSH (p<0.05 to p<0,001) levels as compared to control group.

Table 12: Effect of the petroleum ether and ethanol extracts of *Indigofera tinctoria* on FCA induced rat tissue biochemical parameters

Treatment groups	Tissues				Serum
	SOD (U/mg of tissue)	Catalase (U/mg of tissue)	Glutathione (n mole/mg of tissue)	Lipid peroxidation (n mole/mg of tissue)	Lipid peroxidation (n mole/ml of serum)
Normal	455.7 ± 45.41	0.3570 ± 0.2301	115.4 ± 5.669	699.1 ± 35.02	3090 ± 77.85
Control	101.9±59.82 ***	0.0298±0.0434***	69.34±6.254***	1297 ± 155.0***	1464 ± 113.5***
Diclofenac (10 mg/kg)	209.5 ± 8.635	0.2215 ± 0.0966	131.5 ± 4.183^c	666.6 ± 11.74^c	2867 ± 78.12^c
LPIT (100 mg/kg)	242.0 ± 71.67	0.0454 ± 0.0113	79.95 ± 3.183	977.0 ± 54.59^b	2025 ± 57.40^c
LPIT (300 mg/kg)	363.8 ± 55.89^b	0.0955 ± 0.0118	123.7 ± 8.647^c	659.3 ± 64.22^c	2632 ± 41.90^c
LEIT (100 mg/kg)	215.5 ± 53.45	0.0362 ± 0.0594	81.30 ± 11.62	1149 ± 49.22	732 ± 20.30
LEIT (200 mg/kg)	219.8 ± 6.702	0.0947 ± 0.0638	90.11 ± 6.384	933.5 ± 46.22^b	1866 ± 42.15^a

Values are expressed in mean ± SEM, n=6. One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of p<0.05 was considered as significant. ***p<0.001 as compared to normal group and $^a p$<0.05, $^b p$<0.01, $^c p$<0.001 as compared with control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria* L; LEIT = Leaf ethanol extract of *Indigofera tinctoria* L.

Histamine Level Estimation in Blood and Tissues

In the present study, as shown in figure 2. The concentration of histamine level had significant increase (p<0.001) in the control group. The standard drug Diclofenac and extracts of *Indigofera tinctoria* L had shown the significant inhibition (p<0.05 to p<0.001) of histamine from both blood and liver tissue.

Hematological Parameters and ESR

The hematological parameters like WBC, RBC, hemoglobin (p<0.01), HCT (p<0.05), Lymphocytes and E.S.R (p<0.001) were increased in the control group as compared to normal group. The extracts of *Indigofera tinctoria* L had increased in the WBC, ESR, hemoglobin content (p<0.01), HCT (p<0.05), lymphocytes (p<0.05), ESR (p<0.05 to p<0.001) as shown in Table 13.

IL Measurement

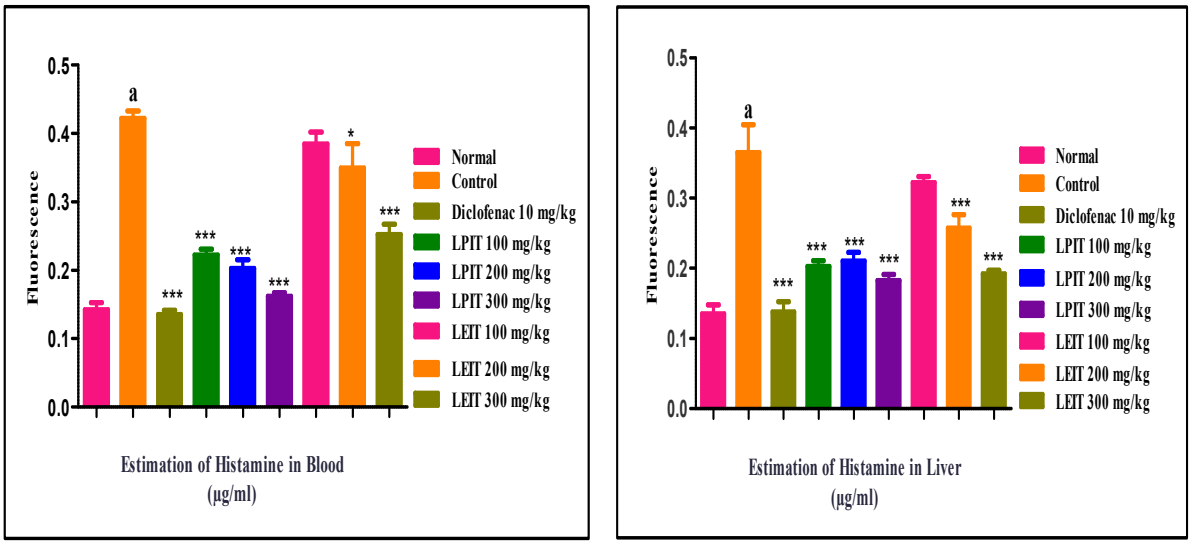
The levels of IL were significantly increased in the control group as compared to normal group. The petroleum ether and ethanol extracts of *Indigofera tinctoria* L had showed significant decrease in the levels of IL as compared to control group.

Radiographic Analysis and Histopathological Assessment

The radiographic (X-ray) analysis of the joints in the arthritis control group shows the soft tissue swelling, bone erosive changes and bone resorption. This indicates the confirmation of arthritis in the ankle region. The extracts treated groups exhibits the reduced swelling in the ankle region (Figure 3) and reduced bone erosive changes and bone resorption as comparison to control. Histopathological studies of indicates significant reduction in the infiltration of leukocytes and disruption and loss of articular damage as compared to control -FCA induced arthritis group, where there is infiltration of leukocytes and articular damage, which is clearly observed (Figure 4).

***In vitro* Proliferation of Spleen Cells (MTT Assay)**

In vitro spleen cell proliferation assay was measured by using MTT to determine cell mediated immunity in FCA induced arthritis. In the control group, there is significant increase (p<0.001) in proliferation of spleenocytes. The standard diclofenac (p<0.001) and extracts of *Indigofera tinctoria* L (p<0.05 to p<0.001) had significant decline the proliferation of spleenocytes as shown in Figure 5.



All values are expressed as mean ± SEM, n=6, One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant; $^{a}p<0.001$ as compared to normal group and $*p<0.05$, $**p< 0.01$, $***p<0.001$ as compared to control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria* L; LEIT = Leaf ethanol extract of *Indigofera tinctoria* L.

Figure 2: Effect of the petroleum ether and ethanol extracts of *Indigofera tinctoria* L on FCA induced histamine level in blood and liver

Table 13: Effect of the petroleum ether and ethanol extract of *Indigofera tinctoria* L on FCA induced rat hematological parameters and E.S.R

Treatment groups	W.B.C ($\times10^3$/µl)	R.B.C ($\times10^3$/µl)	Hemoglobin (g/dL)	Platelets ($\times10^3$/µl)	HCT (%)	Lymphocytes ($\times10^3$/µl)	E.S.R (mm/hr)
Normal	9.50 ± 0.593	7.64 ± 0.11	13.68 ± 0.22	739.5 ± 55.65	44.85 ± 0.54	7.56 ± 0.57	2.20 ± 2.20
Control	13.08± 1.64*	8.03 ± 0.19	14.53 ± 0.14*	725.0 ± 171.0*	46.73 ± 0.60	11.02 ± 1.51	7.20 ± 0.37***
Diclofenac(10mg/kg)	9.81 ± 0.28	7.74 ± 0.16	13.43 ± 0.22	858.5 ± 57.16	44.07 ± 1.03	8.26 ± 0.46	2.40 ± 0.24
LPIT (100 mg/kg)	12.90 ± 1.03	8.03 ± 0.20	14.10 ± 0.27	804.0 ± 15.35	45.33 ± 1.35	10.2 2± 0.78	6.20 ± 0.20
LPIT (200 mg/kg)	10.87 ± 1.08	7.64 ± 0.23	12.5 ± 0.28[b]	74.3 ± 114.1	41.38 ± 1.21	7.93 ± 0.97	5.80 ± 0.20
LPIT (300 mg/kg)	8.16 ± 1.08[a]	8.13 ± 0.30	13.82 ± 0.42	717.7 ± 79.31	45.6 7± 1.74	6.05 ± 0.68[b]	4.00 ± 0.31
LEIT (100 mg/kg)	13.82 ± 1.89	7.65 ± 0.18	13.67 ± 0.25	1090 ± 37.50[a]	42.37± 0.86	12.08 ± 0.87	6.00 ± 0.01
LEIT (200 mg/kg)	11.27 ± 0.88	8.24 ± 0.24	14.53 ± 0.30	971. 8± 79.81	46.23 ± 0.91	8.83 ± 0.91	5.60 ± 0.24
LEIT (300 mg/kg)	9.10 ± 0.55	8.33 ± 0.26	14.08 ± 0.35	729.2 ± 35.18	46.18 ± 0.95	6.45 ± 0.47[b]	5.20 ± 0.37

Values are expressed in mean ± SEM, n=6. One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant. $***p<0.001$ as compared to normal group and $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ as compared with control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria* L; LEIT = Leaf ethanol extract of *Indigofera tinctoria* L.

Table 14: Effect of petroleum ether and ethanol extracts of *Indigofera tinctoria* L on estimation of Interleukin levels

Treatment groups	Results (Pg/ml)	
	Spleen	Serum
Normal	ND (<1.0)	ND (<1.0)
Control	160.111	125.548
Diclofenac (10 mg/kg)	ND (<1.0)	141.255
LPIT (100 mg/kg)	67.87	102.87
LPIT (200 mg/kg)	60.32	98.978
LPIT (300 mg/kg)	31.69	58.42
LEIT (100 mg/kg)	89.65	136.334
LEIT (200 mg/kg)	41.65	11.62
LEIT (300 mg/kg)	ND (<1.0)	ND (<1.0)

ND = Not detectable value is <0.1 pg/ml

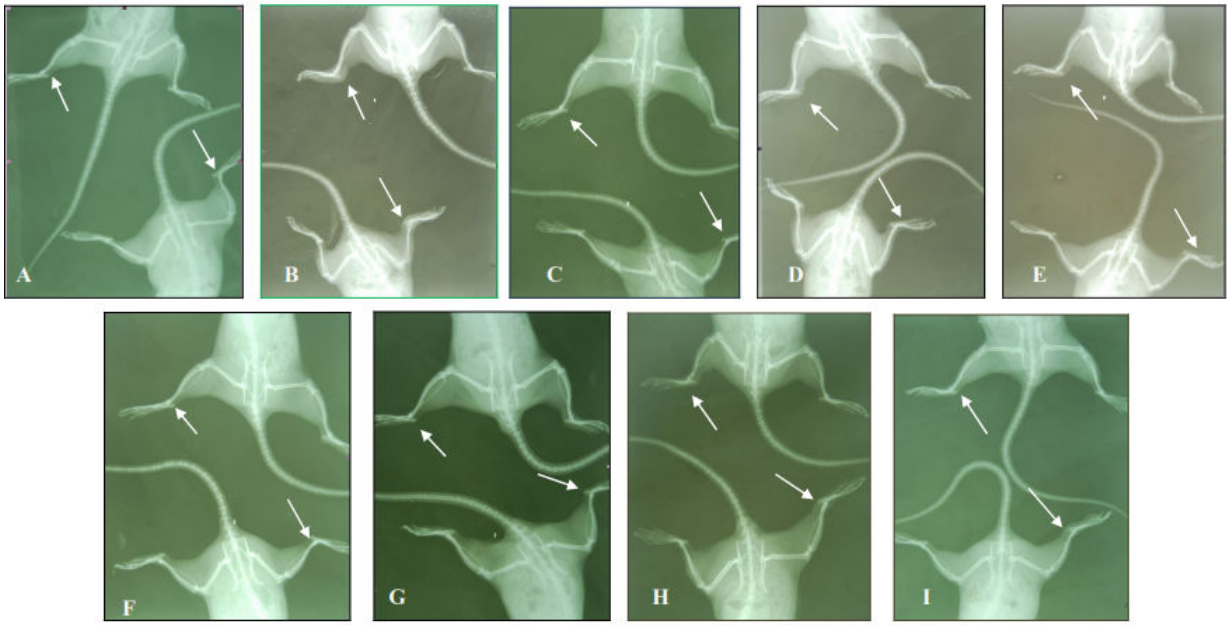


Figure 3: Radiographic changes. **A)** The normal radiographic joint showing no soft tissue swelling and bone erosive changes. **B)** Control group showing the FCA induced arthritis in rats with soft tissue swelling and bone erosive changes. **C)** Diclofenac 10 mg/kg treated animals showing significant reduction in soft tissue swelling and bone erosive changes. **D)** LPIT 100 mg/kg treated animals showing slight reduction in swelling and bone erosive changes. **E)** LPIT 200 mg/kg treated animals showing reduction in soft tissue swelling and joint erosive changes. **F)** LPIT 300 mg/kg treated animals showing reduction in soft tissue swelling and bone erosive changes. **G)** LEIT 100 mg/kg treated animals showing soft tissue swelling and bone erosive changes. **H)** LEIT 200 mg/kg treated animals showing reduced soft tissue swelling with bone erosive changes. **I)** LEIT 300 mg/kg treated animals showing reduced soft tissue swelling and bone erosive changes.

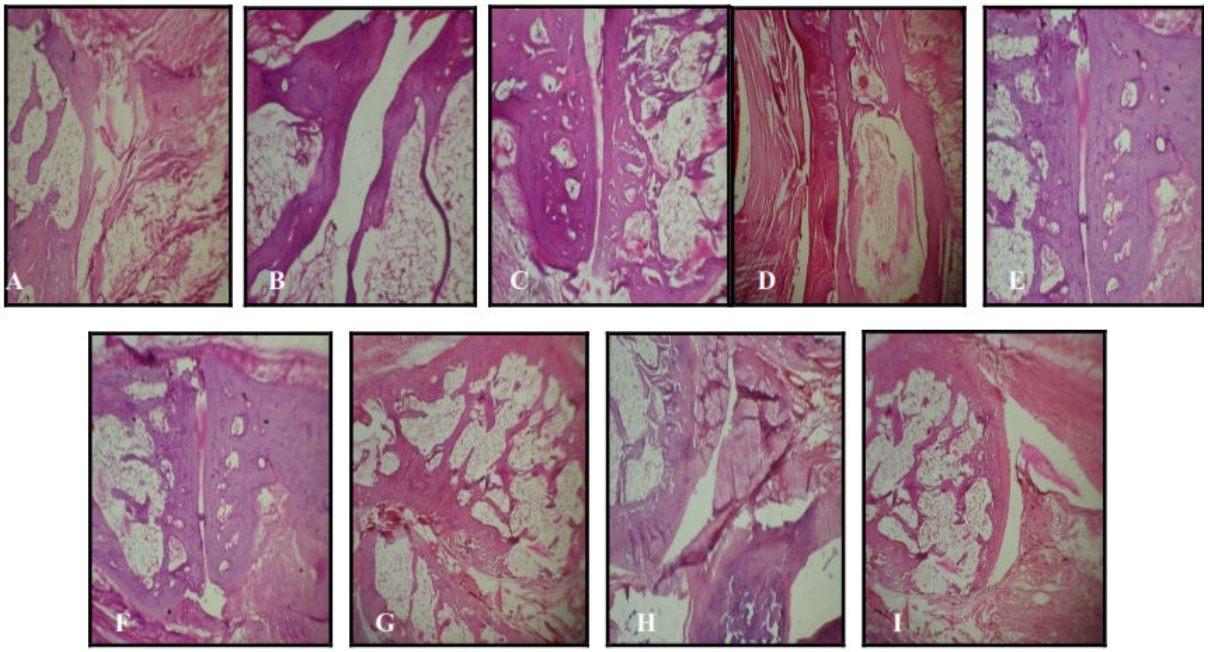
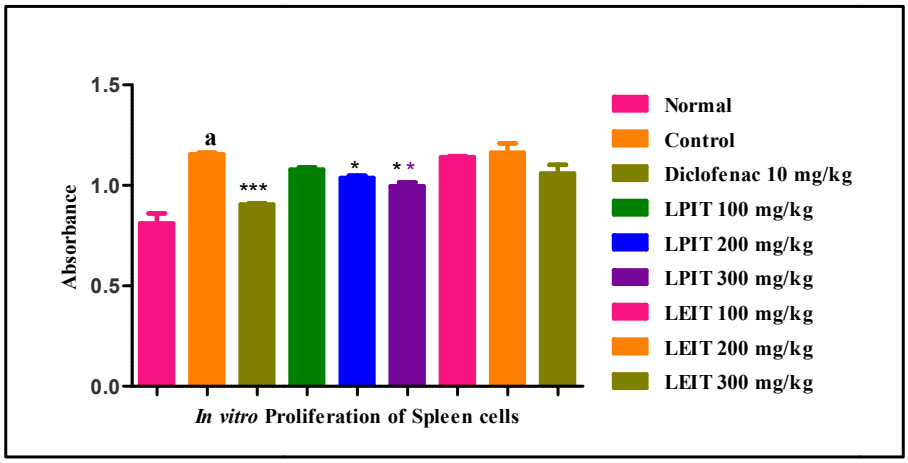


Figure 4: Histopathological changes. **A)** Normal group showing normal cartilage and infiltrate in synovium. **B)** FCA induced control group showing marked infiltration of leukocytes along with the disruption and loss of articular damage. **C)** Diclofenac 10 mg/kg showing decreased articular damage. **D)** LPIT 100 mg/kg showing the marked infiltration, but reduced articular damage. **E)** LPIT 200 mg/kg showing reduced damage on normal cartilage and absence of infiltrate in the synovium. **F)** LPIT 300 mg/kg representing almost as normal group with less articular cartilage damage. **(G)** LEIT 100 mg/kg showing the marked infiltration of leukocytes along with disruption and loss of articular cartilage. **(H)** LEIT 200 mg/kg showing reduced articular damage and marked infiltration of leukocytes. **(I)** LEIT 300 mg/kg showing decreased articular damage and reduced infiltration of leukocytes.



All values All values are expressed as mean ± SEM, n=6, One way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test. The minimum value of $p<0.05$ was considered as significant; $^{a}p<0.001$ as compared to normal group and $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ as compared to control group. LPIT = Leaf petroleum ether extract of *Indigofera tinctoria*; LEIT = Leaf ethanol extract of *Indigofera tinctoria* L.

Figure 5: Effect of the leaf petroleum and ethanol extracts of *Indigofera tinctoria* L on FCA induced arthritis *in vitro* proliferation of spleen cells by MTT assay.

HPLC Analysis of *Indigofera tinctoria* L.

The results of the HPLC analysis of *Indigofera tinctoria* L. were represented in Figure 6. The 4.33 retention time (Rt) indicates the presence of the Indurubicin and the retition time was compared with the standard Indurubicin.

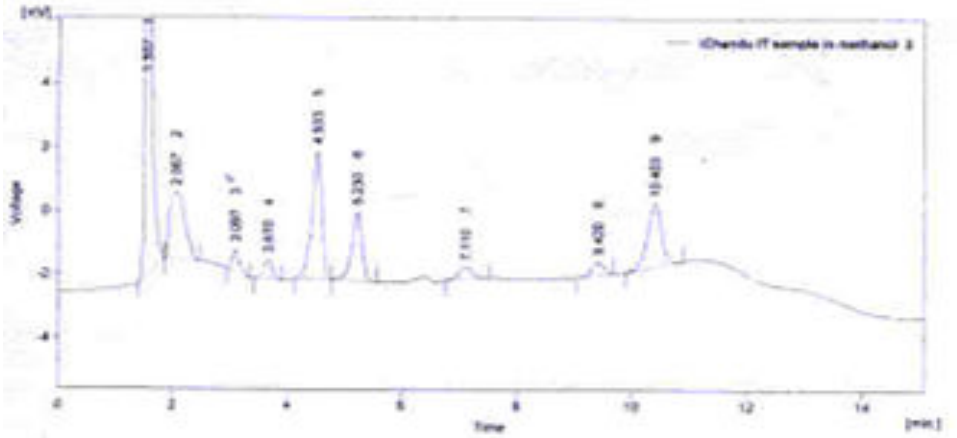


Figure 6: HPLC analysis of *Indigofera tinctoria* and 4.33 retention time (Rt) indicates the presence of the Indurubicin

DISCUSSION

The results of the present study indicates that the leaf extracts of *Indigofera tinctoria* L exhibits the anti-inflammatory against carrageenin induced paw edema and antiarthritic activity against FCA induced acute and chronic models of arthritis. The anti-inflammatory activity by subplantar injection of carrageenin into rat paw produces plasma extravasation and inflammation characterized by increased tissue fluid and plasma protein exudation with neutrophil extravasation and metabolism of arachidonic acid through cyclooxygenase and lipoxygenase enzyme pathways (Szolcsanyi *et al*, 1988; Gamache *et al*, 1986). The edema and inflammation induced by carrageenin is shown to be mediated by histamine and 5-HT during 1st h, after which increased the vascular permeability is maintained by release of kinins up to 2 h, and from 2 to 5th hr the mediators appears to be prostaglandins, the release of which is closely associated with migration of leucocytes into the inflammed site (Dirosa *et al*, 1974). In the present study, the significant decreased paw edema volume in Diclofenac and *Indigofera tinctoria* extract animals might be due to the inhibition of histamine or prostaglandins release by blocking through cyclo-oxygenase pathway.

FCA administered rats showed characteristic of arthritis by soft tissue swelling around the ankle joints during the development of arthritis, which was considered as edema of the particular tissues. This progressed inflammatory leads to more diffused demineralization developed in the extremities. (Begum and Sadique, 1988). The quantitative analysis of behavioral studies in chronic model of adjuvant induced arthritis in rats, can influence the susceptibility to certain diseases, particularly those involving the immune system functions (Berand *et al*, 1987). In the present study, the significant increase in ambulatory movement and rearing, significant decrease in grooming and paw edema might be due to increased demineralization in the joint extremities. Similarly, the Evans blue extravasation method is used to assess for plasma protein extravasation in the knee joint, because Evans blue has high binding affinity to plasma proteins. These large plasma proteins and the bound Evans blue

dye cannot pass through the endothelial gaps and therefore gets restricted in the vascular component. These endothelial gaps get enlarged in the severe arthritis conditions, the plasma proteins and Evans blue dye complex can escape easily through the interstitial tissues. The amount of Evans blue estimation in the synovial capsule can provide us an index of the relative vascular permeability (Franchis *et al*, 2004). In the present study the significant decrease in absorbance in extracts of *Indigofera tinctoria* and diclofenac treated groups, indicates the decreased endothelial groups and decreased infiltration and permeability of inflammatory mediators.

The inflammatory scoring results in the arthritis control group indicates the severe redness, swelling, erythema at the ankle joints, this is due to the release of inflammatory mediators at the localized knee joint, where FCA is administered and in progressively the deformed paw and joint, leading to pannus formation and proliferation of synovium leading to cartilage damage (Tomita *et al*, 2006). The extract treated groups showed significant reduction in the inflammation and cartilage damage, this was supported by decreased swelling, redness, rigidity and pain. The radiographic (x-ray) analysis of the joints in the arthritis control group shows the soft tissue swelling, bone erosive changes and bone resorption. This indicates the confirmation of arthritis. There is restore of the pathological changes to normal significantly in plant treated groups. The histopathological studies revealed that in *Indigofera tinctoria* L showed significant reduction in the infiltration of leukocytes and disruption and loss of articular damage as compared to FCA induced arthritis group, where there is infiltration of leukocytes and articular damage. Cytoplasmic enzymes like ALP, AST, ALT and creatinine serves as indicators of the cellular integrity induced by pathological conditions. These enzymes are used as sensitive markers for evaluation of protective activity, these markers attribute towards persistant inflammation (Narendhirakannan *et al*, 2007). The decreased levels of the above cytoplasmic enzymes supports the protective role of *Indigofera tinctoria* in chronic arthritis. The serum nitric oxide in the control group was increased may be due to increased activity of nitric oxide synthetase (iNOS) or infiltration of macrophages, neutrophils, chondrocytes and fibroblasts during severe arthritic conditions. (Watkins *et al*, 1997; (Stadler *et al*, 1991; Gilchrist *et al*, 2002). The *Indigofera tinctoria* treated extracts showed significant decreased level of NO, indicated the decreased infiltration and may be inhibition of iNOS activity. In RA, the infiltration of neutrophils, macrophages cause the generation of reactive oxygen species (ROS) and through the induction of iNOS, more release of NO results with superoxide anion to form peroxynitrite, a potent reactive molecules capable of eliciting lipid peroxidation (LPO). In the process the oxidative determination of polysaturated lipids leads to release major end product malondialdehyde and this is an index of lipid peroxidation (Kohen and Nyska, 2002). The excessive production LPO causes the imbalance of oxidative homeostasis by decreased protective antioxidant enzymes SOD, CAT and nonenzymatic reducing agents GSH level (Karatas *et al*, 2003; Kamanli *et al*, 2004). The significant reduced LPO and moderate increased level of SOD, CAT and GSH activity indicates the antioxidant property of the extracts.

Rheumatoid arthritis is an inflammatory disease in which the release of histamine through mast cell degranulation and infiltration of neutrophils leads to release of cytokines IL-1, IL-4, IL-18 and their heir mediation plays an important role in progression of arthritis. The significant increase in the histamine level, TNF-α and IL-1β in the control group of present study supports the severe inflammatory conditions (William, 1996). However, the significant decreased histamine release was inhibited by the extracts treated groups, this indicates this plant has mast cell membrane stabilizing property. The decreased level of TNF-α and IL-1β, indicates the suppression of macrophages and neutrophil, indicating the reduced infiltration of inflammatory mediator (Suk-Jong Suh *et al*., 2006). In the present study, the hematological parameters like WBC, RBC, Haemoglobin, MCH, MCHC, HCT, Lymphocytes and E.S.R were increased except the level of MCV and platelets are decreased in the arthritis control group. WBCs that are the major component of the body immune system increases their level during arthritis and inflammatory diseases, due to the release of IL-1β hat stimulates the production of granulocytes and colony stimulating factor (Movat and Semin, 1971; Eric and Lawrence, 1996). E.S.R level, R.B.C and Hemoglobin levels indicate the anemic conditions, which is a common diagnostic feature in patients with arthritis (Eric and Lawrence, 1996). E.S.R is an estimate of susceptibility of the RBC's plasma, which is related to number and size of red blood cells and to the relative concentrations of plasma proteins. The extracts of *Indigofera tinctoria* had shown the protective role against hematological parameters, by decreasing WBC, RBC, hemoglobin, MCH, MCHC and increasing platelets and MCV levels. *In vitro* spleen cell proliferation assay was measured by using MTT to determine cell mediated immunity in FCA induced arthritis. In the control group, there is significant increase in the macrophages in the affected joints that leads to activation of T-cells, that gets involved with the initiation of synovial hyperplasia, which results to bone destruction. T cell infiltrate is directly correlated to severity of arthritis (Tomita *et al*, 2006). The extract of *Indigofera tinctoria* has significantly showed the protective effect against the proliferation of spleen cells, which might be due to decreased activation of macrophages.

CONCLUSIONS

In conclusion, the present finding suggests a potential role of petroleum and ethanol extracts of *Indigofera tinctoria* in FCA induced arthritis. The mechanism by which, this plant acts as antiarthritic activity is probably by inhibiting cyclooxygenase pathway or antioxidant property or through the mast cell membrane stabilizing property. Earlier study has shown alkaloids like indirubin present in extract and flavonoids (Ganapaty *et al*., 2010) exert antiarthritic activity through the above mechanism, this substantiates that alkaloids and flavonoidal content of this plant may be responsible for this activity. Further studies are warranted to pursue molecular mechanism, the interesting lead molecules characterization emerging from this plant results to exploit fall therapeutic potential in arthritic diseases.

Conflict of Interest

All the authors declared no compiting conflict of interest.

Acknowledgement

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