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Study of anti-inflammatory activity of ethanolic extract of *Hemidesmus indicus* roots in acute, subchronic & chronic inflammation in experimental animals

P. Zuber Shaikh
STES's Sinhgad College of Pharmacy , Pune, (MH) - India

Abstract

Hemidesmus indicus, commonly known as "Anantmul," possesses various pharmacological activities including anti-inflammatory activity. The present study is designed to investigate anti-inflammatory effect of ethanolic extract of *Hemidesmus indicus* (EEHI) on rats and mice. The anti-inflammatory effect is investigated employing acute anti-inflammatory models: carrageenan induced paw edema, leukocyte emigration, acetic acid-induced vascular permeability, mast cell degranulation in rats; subchronic anti-inflammatory model: cotton pellet granuloma in mice; and chronic anti-inflammatory models: formailn induced arthritis, delayed type hypersensitivity using egg white lysozyme as an antigen. EEHI (100, 200 mg/kg, p.o) exhibited a dose-dependent and significant inhibition (P <0.05) in all the experimental models. Preliminary phytochemical screening revealed the presence of flavonoids, steroid, terpenoid, saponins, tannins,. The extract produces no mortality in the dose up to 2000 mg/kg, p.o. The results obtained suggest marked anti-inflammatory activity of the EEHI and support the traditional use of this plant in inflammatory conditions.

Key-Words: *Hemidesmus indicus*, Vascular permeability, Granuloma, Hypersensitivity, Edema

Introduction

Inflammation is a physiological process in response to tissue damage resulting from microbial pathogen infection, chemical irritation, & / or wounding.[1] The relation between inflammation & atherosclerosis, diabetes, cancer, arthritis & alzheimer's disease has been well substantiated.[2] The functioning of the immune system is finely balanced by the activities of pro-inflammatory & anti-inflammatory mediators of inflammation. The mediators of the inflammation such as cytokines, eicosanoids & free radicals have direct or indirect effect on the pathophysiology of diseases. Chronic inflammation develops from unresolved symptomatic acute inflammation with or without any clinical manifestations. This may activate macrophages & lymphocytes which release inflammatory mediators. Neutrophils are the first blood leukocytes to arrive to an inflammatory site. The process of transendothelial migration from the blood to the tissue is complex & involves response to chemotactic factors & binding to adhesion molecules at the endothelium surface.[6]

*** Corresponding Author**
E.mail: zuby.shaikh56@gmail.com;
zubyshaikh20@gmail.com.
Tel..:020-24373961.

These events permit the extravasation of blood neutrophils to the tissue where they can perform different functions. Major roles of neutrophils include phagocytosis, production of reactive oxygen & nitrogen species that damage DNA & cell membranes & production of chemoattractants such as IL-8 which recruit further competent cells.[7] Neutrophil response usually terminates when neutrophils undergo apoptosis & are phagocytosed by arising macrophages.[8] Inflammatory cells release prostaglandins with concomitant increase in the expression of key enzyme cyclooxygenase which in turn can activate several transcription factors including NF-κB.[9] Inflammation activates a variety of inflammatory cells, which induce & activate oxidant generating enzymes like NADPH oxidase, xanthine oxidase, myeloperoxidase, etc.; which produce superoxide anion & other reactive nitrogen species like nitric oxide through activation of inducible nitric oxide synthase (iNOS).[10] Free radicals play major role in persistence of inflammation. During the process of inflammation, inflammatory cells secrete chemically reactive oxidants, radicals & electrophilic compounds that bing abou the elimination of the infectious agents.[11-12] These inflammatory mediators can damage the surrounding host tissue.[13-14] Many

drugs of plant origin having antioxidant activity have been reported to have anti-iflammatory activity.[15-16] Though it is a defense mechanism, the complex events & mediators involved in the inflammatory reaction can induce, maintain or aggravate many diseases.[17] However, studies have been continuing on inflammatory diseases & the side effects of the currently available anti-inflammatory drugs pose a major problem during their clinical use .[18] Hence, the development of newer & more powerful anti-inflammatory drugs with lesser side effects is necessary.

The roots of *Hemidesmus indicus* have been used in Indian folk medicine to treat various inflammatory conditions. Other traditional uses are astringent, immunosuppressant and also used in hepatic and renal disorders (toxicity).[19] Though the plant and its extracts have been used in the folklore medicine extensively, there is no scientific evidence for such activities available in established scientific journals of repute. Keeping this in view, the present study has been undertaken to investigate the anti-inflammatory potential of ethanolic extract of Hemidesmus indicus (EEHI) on carrageenan induced paw edema, leukocyte emigration ,acetic acid-induced vascular permeability, mast cell degranulation in rats cotton pellet-induced granuloma in mice, formalin induced arthritis & delayed type hypersensitivity using egg white lysozyme as an antigen in rats.

Material and Methods

Collection & identification of plant material

The roots of *Hemidesmus indicus* were collected at Pune , Maharashtra , India. The roots were macroscopically, organoleptically & chromatographically identified by Smt A. S. Upadhye, Scientist, Plant Drug Authentication Service, Botany Group, Plant Sciences Division (Auth10-06).

Preparation of plant extraction

The collected roots *of Hemidesmus indicus* were dried at 400C in an oven and reduced to coarse powder using a mechanical grinder. The powdered material of the roots was exhaustively extracted with (95%) ethanol under the maceration process. The macerated mixture was filtered and evaporated under reduced pressure to yield a reddish brown solid extract. The extract was stored in a desiccator and dilutions of the extract were made in 2% gum acacia or any suitable suspending agent for pharmacological studies.

Preliminary phytochemical screening

The ethanolic extract was screened for the presence of various phytoconstituents like alkaloids, steroids, glycosoides, triterpenoids, tannins, flavonoids & saponins by employing standard phytochemical tests.[20]

Physicochemical properties of EEHI

The ethanolic extract was observed for appearance (i.e. state, colour, odour & taste, etc.), solubility, ash values, Ultraviolet absorbance (ε_{max}) at 280 nm (for flavonoids & tannins) & for HPTLC profile at 280nm the procedure for which as follows:

High performance thin layer chromatography (HPTLC) analysis of EEHI (Sircar et al. 2006).

Sample preparation: EEHI 100 mg was dissolved in 10ml of ethanol and sample of 5 µl , 10 µl and 20 µl were applied as 6 mm wide bands, under a continuous flow of nitrogen, using CAMAG LINOMATE V automatic sample applicator. Sample was applied with a 100 µl syringe (Hamilton, Bonaduz, Switzerland) at a constant application rate of 0.1µl/s and the distance between adjacent bands was 10 mm.

A. Stationary phase: The 05 x 10 cm aluminum-backed HPTLC plate coated with 250 µm layers of Silica gel G 60 F254 (E. Merck, Darmstadt, Germany; supplied by Merck India, Mumbai, India) was prewashed with methanol and activated at 110^{o}C for 10 min was used as stationary phase.

B. Mobile phase: Chloroform: Methanol [9.2: 0.8 (v/v)] was used as mobile phase.

C. Development: The plate was developed in an ascending manner with a solvent system consisting of Chloroform: Methanol [9.2:0.8 (v/v)] in a development chamber pre-saturated with the mobile phase. Developing distance was 1 cm from lower edge of the plate. The length of each chromatogram run was 8cm. The developed plates were air dried.

D. Densitometer scans: Plate was scanned at 280 nm using scanner-3 (CAMAG) operated in reflectance–absorbance mode and controlled by Win CATS software (Version 1.4.3). The slit dimensions were 5 x 0.45 mm and the scanning speed was 20 mm/s. The source of radiation used was deuterium lamp emitting continuous UV spectra between 190-400 nm.

E. Derivatization: Plates were derivatized in Anisaldehyde- H_2SO_4 reagent.

F. Documentation: The profile obtained was video documented at 280 nm and visible before and after derivatization of the plate.

Animals

Male Sprague Dawley rats & Swiss Albino mice of weighing 150-200g &18-22g , respectively were purchased from Intox Pvt.Ltd, Pirangut-Pune. Animals were placed separately in polypropylene cages (five per cage) randomly with paddy husk as bedding. The animals were maintained under standard laboratory

conditions at temperature 23 ± 2^0C, relative humidity 55 ± 10 % and 12 h light and dark cycle throughout all the experiments. Animals had free access of water and standard laboratory feed. The animals were shifted to the laboratory one hour prior to the experiment. All animals were acclimatized for at least 1 week before the experimental session. All the experimental procedures were done following the guidelines of Institutional Animal Ethics Committee (IAEC).

Drugs and chemicals

Acetic acid (Poona chemical laboratory, Pune), Carrageenan (Analab fine chemicals, Mumbai), Compound 48/80 (Poona chemical laboratory, Pune), Diclofenac sodium (Cure medicines (I) Pvt.Ltd. Pune.), Evans blue (Sigma, St. Louis, MO, USA), Formalin (Poona chemical laboratory, Pune). All other chemicals were of analytical grade and procured locally.

Acute toxicity study

Acute oral toxicity study was performed in mice by following Organization for Economic Co-operation & Development guidelines AOT No. 425. Healthy adult albino mice (18- 22g) were subjected to acute toxicity studies as per guidelines (OECD 425) suggested by the OECD. The mice were observed continuously for 3 h for behavioral and autonomic profiles and for any sign of toxicity or mortality up to a period of seven days. (OECD Guideline 425)

Experimental design

Four groups were employed in the present anti-inflammatory study. Each group consist of 5 rats or mice.

Group I- Inflammation control,

Group II- Inflammation treated 10mg/kg of Diclofenac sodium (Standard),

Group III- Inflammation treated 100mg/kg of EEHI (Test-I),

Group IV- Inflammation treated 200mg/kg of EEHI (Test-II),

Carrageenan induced paw edema

The carrageenan induced paw edema was executed according to methods described.[21-22] Rats (150-200g) were divided into 4 groups of 5 animals each. Rats overnight fasted were administered with EEHI (100mg/kg & 200mg/kg), Diclofenac sodium (10mg/kg) & vehicle (0.5% CMC) orally to group Test-I, Test-II, Standard & Inflammation control, respectively 1hr before subplantar administration of 0.1ml of 1% w/v carrageenan (in normal saline) in the right hind paw. The left paw receive same volume of normal saline.

A) For Paw volume

The paw volume was measured at 0, 1, 2 & 3 Hr using plethysmometer. The increase in the paw volume of both the groups over a period of 3 hrs. was calculated & compared with standard.

B) For paw thickness

The paw thickness was measured at 0, 1, 2 & 3 Hr using Vernier calipers. The increase in the paw thickness of both the groups over a period of 3 hrs. was calculated & compared with standard.

Leukocyte emigration & other haematological parameters

The leukocyte emigration & other haematological parameters was executed according to methods described.[23-25] Rats (150-200g) were divided into 4 groups of 5 animals each. Rats overnight fasted were administered with EEHI (100mg/kg & 200mg/kg), Diclofenac sodium (10mg/kg), vehicle (0.5% CMC) orally to group Test-I, Test-II, Standard & Inflammation control, respectively 1hr before intrapleural administration of 0.25ml of 1% Carrageenan in normal saline. Five hrs after carrageenan injection, the intrapleural exudates was collected & measured & total leukocyte count was made. & other Haematological parameters like RBC Count, Erythrocyte sedimentation rate, Haemoglobin content were measured & test group values are compared with standard group values.

Acetic acid induced vascular (capillary) permeability method

The acetic acid induced vascular (capillary) permeability was executed according to method described.[26] Rats (150-200g) were divided into 4 groups of 5 animals each. Rats overnight fasted were administered with EEHI (100mg/kg & 200mg/kg), Diclofenac sodium (10mg/kg), vehicle (0.5% CMC) orally to group Test-I, Test-II, Standard & Inflammation control, respectively 1hr before the injection of 0.25 ml of 0.6% solution of acetic acid intraperitoneally. Immediately after administration, 10 mg/kg of 10% (w/v) Evan's blue is injected intravenously through the tail vein. Thirty minutes after Evan's blue injection, the animals are hold by a flap of abdominal wall and the viscera irrigated with distilled water over a petridish. The exudate is then filtered and makes the volume made up to 10 ml. The dyes leaking out into the peritoneal cavity measured spectrophotometrically at 620 nm and compared with the control group.

Mast cell degranulation

The mast cell degranulation was executed according to method described.[27] Rats (150-200g) were divided into 4 groups of 5 animals each. Rats overnight fasted were administered with EEHI (100mg/kg & 200mg/kg), Diclofenac sodium (10mg/kg), vehicle (0.5% CMC) orally to group Test-I, Test-II, Standard & Inflammation control, respectively 1 hr before the injection of compound 48/80 (3µg in 1 ml of normal saline solution), intraperitonially. The rats were killed by asphyxiation in CO_2 followed by cervical dislocation, and the peritoneal cells were recovered by lavage of the peritoneal cavity with 100 ml of buffer solution. The cells were sedimented by centrifugation, washed and resuspended in 3ml of buffer solution. Cells were maintained on ice throughout. Cytospins of the cells were prepared, fixed in methanol and then stained in 5% Giemsa for 20min. The % inhibition of degranulation and total mast cells were counted by light microscopy by an observer & compared with standard.

Cotton pellet granuloma in mice

The Cotton pellet granuloma in mice was executed according to method described.[28] Mice (18-22g) were anaesthetized with ether (anaesthetic ether I.P.) sterile cotton pellet (10mg) was inserted in the subcutaneous layer of groin of each mice. The incised skin was properly sutured & a disinfectant was applied to prevent infection. EEHI (100mg/kg & 200mg/kg) & standard (Diclofenac sodium) 10mg/kg, vehicle (0.5% CMC) were orally administered to group Test-I, Test-II, Standard & Inflammation control, respectively, for seven days. The mice were sacrificed on the 7^{th} or 8^{th} day by an overdose of ether. The cotton pellets were removed, dried at 37^{o} C for 24 hrs. & weighed. The results were expressed as the difference between initial weight (10mg) & the final dry weight of the cotton pellets & also expressed as % inhibition of granuloma.

Formalin induced arthritis

The formalin induced arthritis was executed according to methods described.[29-30] Radiographic analysis was executed according to methods described.[31] Rats (150-200g) were divided into 4 groups of 5 animals each. Rats overnight fasted were administered with EEHI (100mg/kg & 200mg/kg), Diclofenac sodium (10mg/kg) & vehicle (0.5% CMC) orally to group Test-I, Test-II, Standard & Inflammation control, respectively, for eight days. Inflammation was produced by subplanter injection of 0.1 ml of 2% w/v formalin in normal saline in the right hind paw of the rats on the first & third day.

A) For Paw volume

The paw volume was measured at daily using plethysmometer. The increase in the paw volume of both the groups over a period of 8 days was calculated & compared with standard.

B) For paw thickness

The rat paw thickness was measured daily for 8 days using Vernier calipers. The increase in the paw thickness of both the groups over a period of 8 days was calculated & compared with standard.

C) For radiographic analysis

Male Sprague dawley rats was sacrificed on 21st day of formalin administration and legs are removed and placed on formalin containing plastic bag. This plastic bag was kept at a distance of 90 cm from the X-ray source was kept at radiographic analysis of normal and arthritic rat hind paws was performed by X-ray machine with a 300-mA exposition for 0.01 s.

D) For histopathological study

A portion of joint tissue from each group was preserved in a 10% formaldehyde solution for histopathological studies. Haematoxylin & Eosin were for staining & later the microscopic slides of the joint tissue were photographed at a magnification of 100X.

Delayed type hypersensitivity

The delayed type hypersensitivity was executed according to method described.[32] Rats (150-200g) were divided into 4 groups of 5 animals each. Rats overnight fasted were administered with EEHI (100mg/kg & 200mg/kg), Diclofenac sodium (10mg/kg) & vehicle (0.5% CMC) orally to group Test-I, Test-II, Standard & Inflammation control, respectively, for eight days, 1^{st} hr before antigen sensitization on 1^{st} day of the experiment. All the rats were immunized (i.p.) on day 0 with 0.1ml of egg white. The edema was induced in the right hind paw of rats by challenging with 0.1 ml of egg white into the subplanter region on day 7. The contralateral paw received equal volume of saline & was served as a control.

A) For Paw volume

The increased in the paw volume at 0 & 48 hr was assessed using a plethysmometer. The increase in the paw volume of both the groups over a period of 2 days (7^{th} & 8^{th} day) was calculated & compared with standard.

B) For paw thickness

The rat paw thickness was measured daily for 2 days using Vernier calipers. The increase in the paw thickness of both the groups over a period of 2 days (7^{th} & 8^{th} day) was calculated & compared with standard.

Statistical Analysis

The experimental results were expressed as the mean ± SEM. Data were assessed by the method of analysis of variance followed by Dunnett's *t*-test. *P* value of <0.05 was considered statistically significant.

Results and Conclusion

Preliminary phytochemical screening of EEHI

The preliminary phytochemical screening showed presence of saponins, steroids, triterpenoids, tannins and flavonoids in ethanolic extract of roots of *Hemidesmus indicus*.

High Performance Thin Layer Chromatography (HPTLC) analysis of EEHI:

1) P-methoxy salicylic aldehyde;
2) Hemidesmine;
3) Flavonoids;
4) Triterpenoids;
5) 2-methoxy-4-hydroxy benzaldehyde (Tannins);
6) Steroids;
7) Saponins.

Acute Toxicity Study

In the acute toxicity study, no mortality was observed during the seven days period at the dose tested (2000mg/kg) & the animals showed no stereotypical symptoms associated with toxicity such as convulsion, ataxia, diarrhea or increased diuresis during initial three hours period. From this data, two different doses 100 & 200 mg/kg were selected for further study**.**

Carrageenan induced paw edema in rats

After oral treatment with EEHI (100 & 200mg/kg) & Diclofenac sodium 10mg/kg, it is observed that EEHI is more effective in reducing the paw volume & paw thickness than Diclofenac sodium, after induction of carrageenan induced paw edema in rats.

Leukocyte emigration in rats & other haematological parameters

By comparing the effect of various doses of EEHI & Diclofenac sodium on various haematological parameters of rat pleural blood is as follows:

Total leukocyte count (TLC): Diclofenac sodium 10 mg/kg shows significant decrease in TLC as compared to EEHI (100 & 200mg/kg).

Red Blood Cell Count (RBC): EEHI (100 & 200mg/kg) significantly controls the level of RBC near to the normal range as compared to the Diclofenac sodium 10 mg/kg.

Haemoglobin (Hb gm%): EEHI (100 & 200mg/kg) significantly controls the level of Haemoglobin near to the normal range as compared to the Diclofenac sodium 10 mg/kg.

Erythrocyte Sedimentation Rate: EEHI (100 & 200mg/kg) significantly controls the level of ESR near to the normal range as compared to the Diclofenac sodium 10 mg/kg.

Acetic acid induced vascular (capillary) permeability method in rats

The vascular permeability test is one of the acute inflammatory models. The dye leakage induced by acetic acid was significantly inhibited by 26.1%, 21.9% & 39.5% in response to EEHI (200 & 100mg/kg) & 10mg/kg of Diclofenac sodium, respectively (compared with the control group). The anti-inflammatory activity of EEHI was less effective than the standard drug. However, the anti-inflammatory effect was statistically significant compared with the control group.

Mast cell degranulation in rats

After oral treatment with EEHI (100 & 200mg/kg) & Diclofenac sodium 10mg/kg, it is observed that EEHI is more effective in percent (%) inhibition of mast cell degranulation & in reducing total number of mast cells than Diclofenac sodium, after intraperitoneal administration of compound 48/80 in rats.

Cotton pellet granuloma in mice

Cotton pellet induced chronic inflammatory response characterized with granuloma formation, fluid infiltration & undifferentiated connective tissue was measured by weighing the dried pellets after 8 days of implantation & treatment. The weight of the granuloma for the control group of animals was found to be 85.00 ± 2.51mg. Treatment with EEHI at 100 & 200 mg/kg p.o. decreases the granuloma weight to 66.00 ± 1.08 mg & 62.33 ± 1.76, respectively. Treatment with Diclofenac sodium 10mg/kg p.o. decreased the granuloma weight 68.67 ± 1.76mg. Thus, from the above observation, as mentioned in table, it was postulated that EEHI is more effective than Diclofenac sodium in reducing the granuloma formation.

Formalin induced arthritis in rats

Continuous oral treatment (8 days) with EEHI (100 & 200mg/kg) & Diclofenac sodium 10mg/kg, it is observed that EEHI is more effective in reducing the paw volume & paw thickness than Diclofenac sodium, after 1st & 3rd day of arthritis induction.

Radiographic analysis

Control animal (Arthritis) showing soft tissue swelling with diffused joint in phalangeal region, bending of phalangeal joints and narrowing of joint space were observed. EEHI showing no narrowing of joint space and resembling near normal radiographic pattern of the joints.

Histopathological changes

Histological changes in joints of experimental animals. Section of joint cavity of arthritis rats showing proliferation with granulation tissue adjacent to the

damaged articular cartilage. Section of joint cavity of arthritis rats showing mononuclear cell infilteration.
Section of joint cavity of EEHI & Diclofenac sodium treated rats showing normal architecture of both cartilages with no granulation tissue seen but only small fibrous strands in between the cartilage was observed.

Delayed type hypersensitivity in rats

Continuous oral treatment (8 days) with EEHI (100 & 200mg/kg) & Diclofenac sodium 10mg/kg, it is observed that EEHI is more effective in reducing the paw volume & paw thickness than Diclofenac sodium, after (1^{st} & 7^{th} day of immunization & sensitization) induction of delayed type hypersensitivity.

In the present study, the anti-inflammatory activity of the ethanolic extract of *H. indicus* has been evaluated using both acute and chronic inflammatory models. The experimental models of inflammation are produced by different agents by releasing different types of inflammatory mediators. Each is known to elicit distinct mechanisms of action for producing inflammation by increased in vascular permeability, the infiltrations of leukocytes from the blood into the tissue or granuloma formation and tissue repair. Among the many methods used for screening of anti-inflammatory drugs, one of the most commonly employed techniques is based upon the ability of such agents to inhibit hind paw edema of the rat after the injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, Aerosil®, sulfated polysaccharides like carrageenan or naphthoylheparamine. For inducing edema, histamine, xylene, arachidonic acid, phorbol myristate acetate, oxozolone, croton oil and formalin are also used.

For evaluating the most effective and widely used model for inflammation is carrageenan-induced paw edema, Carrageenan is a mixture of polysaccharides composed of sulfated galactose units and is derived from Irish Sea moss, Chondrous crispus. Its use as an endemogen was introduced by Winter et.al..[33]Carrageenan is known to release histamine & serotonin followed by prostaglandins, protease & lysosomal enzymes during edema formation along with migration of leukocytes & interleukins. Angiogenesis, nitric oxide synthesis & kinin release are found to be the main causes of granuloma formation. Angiogenesis in chronic inflammatory state, facilitates migration of inflammatory cells into the inflammatory site & supplied nutrients & oxygen to tissue. Hence by suppressing angiogenesis in granulation tissue, it is possible to reduce / control the development of chronic granulation tissue (Ghosh et al. 2000). The rate limiting enzyme, nitric oxide synthase is highly induced during inflammation thereby facilitating the formation of nitric oxide which in turn elicit cellular injury.

Kinins cause vasodilatation, increase vascular permeability and WBC migration in the early stages of the inflammation and are also involved in, collagen formation in the later stages of inflammation. Kinins degranulate mast cells to release histamine as well as other mediators of inflammation and cause plasma extravasation by contraction of vascular endothelial cells. Kinins are potent algogenic substances, which induce pain by directly stimulating nociceptors in skin joint, and muscles.[34]

The carrageenan-induced paw edema model in rats is known to be sensitive to cyclooxygenase inhibitors and has been used to evaluate the effect of non-steroidal anti-inflammatory agents, which are known to inhibit the cyclooxygenase thereby decrease prostaglandin synthesis.[35] The time course of edema development in carrageenan-induced paw edema model in rats is generally represented by a biphasic curve.[36] The first phase of inflammation occurs within an hour of carrageenan injection and is partly due to the trauma of injection and also to histamine and serotonin component.[37] Prostaglandins (PGs) play a major role in the development of the second phase of inflammatory reaction which is measured at 3 h.[38a-b] The presence of PGE_2 in the inflammatory exudates from the injected foot can be demonstrated at 3 h and period thereafter. Therefore, it can be inferred that the inhibitory effect of EEHI on carrageenan-induced inflammation could be due to inhibition of the enzyme cyclooxygenase leading to inhibition of prostaglandin synthesis. Significant inhibition of paw edema in the early hours of study by EEHI could be attributed to the inhibition of histamine and/or serotonin. Although EEHI exhibited significant anti-inflammatory activity, the exact mechanisms underlying the observed pharmacological effects can only be elucidated after isolation of active constituents using a wide range of experimental models.[39]

Intrapleural injection of carrageenan leads to inflammation of the pleura resulting from macrophages in the carrageenan insulated tissue. Interleukin-1, a proinflammatory cytokine, induces accumulation of PMN cells by a variety of processes including adhesion & cell mobility.[40] Leukocyte aggregation is a major event during inflammation. The cell migration occurs as a result of much different process including adhesion & cell mobility.

Acetic acid induced vascular permeability rat model is routinely used for determining inflammatory response of vascular tissue origin. EEHI treatment reduced the

intensity of the peritoneal inflammation produced by acetic acid, suggesting its ability. This point out that EEHI has the ability to inhibit the permeability of small blood vessels during acute inflammation.[41] In acetic acid induced vascular permeability, acetic acid causes dilation of arterioles and venules and increased vascular permeability by releasing inflammatory mediators such as histamine, prostaglandins and leukotrienes are released following stimulation of mast cells.

Compound 48/80 demonstrating that the freshly isolated mast cells were not generally unresponsive to secretory stimuli. The acquisition by mast cells of secretory responsiveness was accompanied by a significant increase in expression of the compound 48/80. However, the cells level of expression of plasma membrane-bound IgE remained constant (84-92% of positive mast cells), suggesting that the acquisition of enhanced responsiveness to anti-IgE certainly was not related to changes in their surface IgE expression to the actions of compound 48/80 were essentially unaltered. Compound 48/80 trigger secretion by activating G proteins directly. This suggests that receptor-dependent (IgE-mediated) but not receptor-independent secretory mechanisms during mast cell degranulation. Accordingly, it is proposed that the effect of long-term degranulation on the mast cells responsiveness to receptor-dependent activation probably involves elements of the secretory machinery which come into play prior to Ca^{2+} mobilization or the activation of compound 48/80-inducible G proteins.[42-44]

In cotton pellet induced granuloma when cotton pellet implanted subcutaneously leading to the formation of granular tissue. The amount of granular tissue is measured by weighing the dried pellet after removal as an index of the extended severity of the inflammation. This model involves the proliferative phase of the inflammation of the microphages, neutrophils, fibroblasts and collagen formation which are basic source for the granuloma formation; therefore decrease in the granuloma formation indicates the suppression of the proliferative phase. The cotton pellet granuloma method is widely used to evaluate the transudative and proliferative components of the chronic inflammation.[45] The wet weight of the cotton pellet correlates with the transuda; the dry weight of the pellet correlates with the amount of the granulomatous tissue.[46] Administration of EEHI and diclofenac appears to be effective in inhibiting the wet weight of cotton pellet. On the other hand, the EEHI effect on the dry weight of the cotton pellet was almost near to that of diclofenac. These data support the hypothesis of the greater effect of the inflammation mediators in the immediate response of inflammation. This effect may be due to the cellular migration to injured sites and accumulation of collagen.

The development of arthritis in the rat paw (phalangeal joints) after the injection of formalin is a biphasic event. The initial phase of the edema is due to the release of histamine and serotonin and the edema is maintained during the plateau phase by kinin-like substance [47] and the second accelerating phase of edema formation may be due to the release of prostaglandin-like substances. Inhibition of edema observed in the formalin model may be due to the ability of EEHI to inhibit these chemical mediators of inflammation.[48] It is well known that inhibition of edema induced by formalin in rats is one of the most suitable test procedures to screen anti-arthritic and anti-inflammatory agents, as it closely resembles human arthritis.[49] Arthritis induced by formalin is a model used for the evaluation of an agent with probable antiproliferative activity. The results of the formalin induced arthritis ruled out a possible effect of the extract on formalin induced cell damage and accordingly, arthritic conditions.[50]

Many of the disorders today are based on the imbalances of immunological processes like **DTH** (cell mediated) reactions and humoral responses.DTH is a part of the process of graft rejection, tumour immunity and also immunity related to many intracellular infectious micro-organisms, especially those causing chronic diseases viz tuberculosis. Further, DTH requires the specific recognitions of a given antigen by activated lymphocytes which subsequently proliferate and release cytokines. These in turn, increase vascular permeability, induce vasodilation, macrophage accumulation and activations, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective repair process. In the present, DTH reaction is measured by foot-pad thickness, after 48hr of antigenic challenge and subsequent immunization with egg white, the animal showed significant increase in volume of paw edema due to inflammation in response to the antigen. This potentiation of DTH response indicates that EEHI has stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction and thus increases cell mediated immunity.[51]

The presence of phytoconstituents specially flavonoids & tannins in the root extract may contribute to its observed anti-inflammatory activity. Many flavonoids have been found to exhibit anti-inflammatory effects.

In conclusion, the results of the present study support to the traditional use of *H.indicus* root extract (EEHI), possessing significant anti-inflammatory activity. This

may be due to the presence of triterpenoids, saponins & tannins which deserves further studies to establish its therapeutic value as well as its mechanism of action.

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Table 1: Physicochemical properties of EEHI

Sr. No.	Physicochemical properties		Observation
1)	Appearance		
	a)	State (Nature)	Semisolid
	b)	Color	Dark reddish brown
	c)	Odour	Charactristic & Aromatic
	d)	Taste	Charactristic & Aromatic
2)	Solubility		
	a)	Chloroform	Soluble
	b)	n-Hexane	Soluble
	c)	Methanol	Soluble
3)	Ash value		
	a)	Total ash value	3.5%
	b)	Acid insoluble ash values	16%
4)	Ultraviolet absorbance (ε_{max}) at 280 nm		
	a)	Flavonoid	0.1242
	b)	Tannin	0.0089

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Table 2 : High Performance Thin Layer Chromatography (HPTLC) analysis of EEHI:

Peak	Start Position (R_f)	Start Height (AU)	Max. Position (R_f)	Max. Height (AU)	Max. %	End Position (R_f)	End Height (AU)	Area (AU)	Area %
1	0.00	2.1	0.01	141.1	14.49	0.03	0.0	912.5	3.21
2	0.12	0.1	0.16	26.7	2.74	0.18	2.8	525.4	1.85
3	0.19	18.8	0.21	42.9	4.41	0.25	0.5	885.1	3.11
4	0.26	0.3	0.31	69.9	7.18	0.35	0.0	1748.5	6.15
5	0.36	2.8	0.43	409.1	42.02	0.47	89.7	13231.7	46.55
6	0.47	89.9	0.49	127.6	13.10	0.55	2.0	3997.7	1.68
7	0.65	0.0	0.75	156.3	1.57	0.41	0.3	390.4	1.02

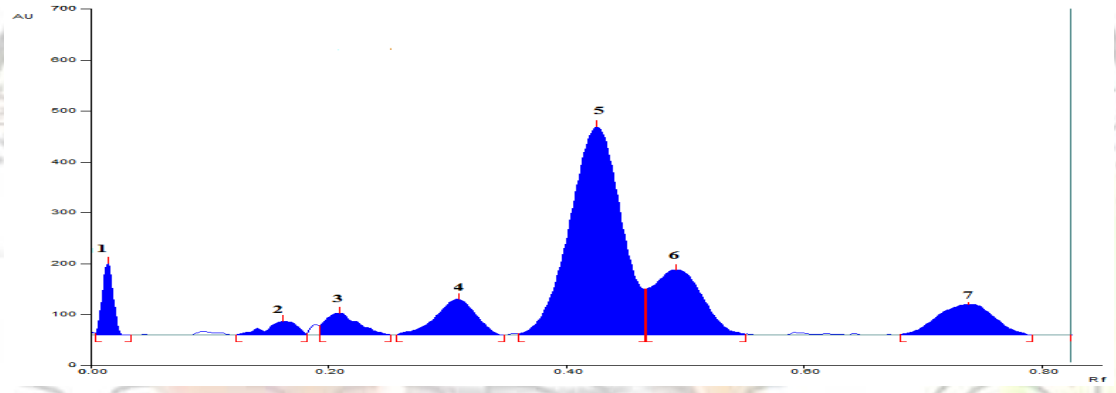
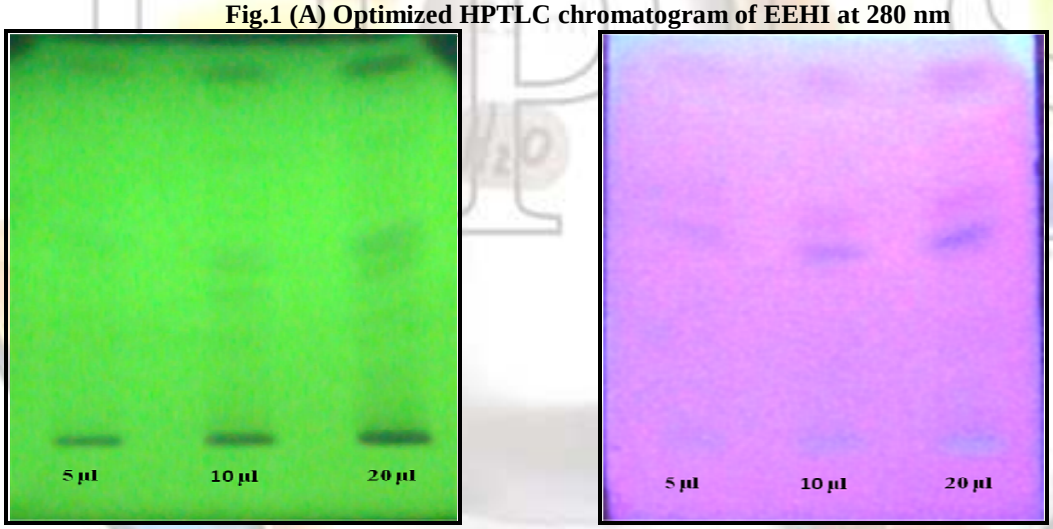


Fig.1 (A) Optimized HPTLC chromatogram of EEHI at 280 nm



(a) **(b)**

Fig.1 (B) Optimized HPTLC chromatogram of EEHI (a) Before derivatization and (b) After derivatization

Carrageenan induced paw edema in rats.

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Table 3 (A): Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using carrageenan induced paw edema in rats: Paw volume

Time interval (hours)	Paw volume (ml) Mean ± SEM (n=5)							
	Inflammation Control		Inflammation Standard		Inflammation Test-II		Inflammation Test-II	
	Control	Inflamed	Control	Inflamed	Control	Inflamed	Control	Inflamed
'0' hr	1.74 ± 0.06	2.61 ± 0.18	1.75 ± 0.07	2.12 ± 0.12	1.59 ± 0.13	2.15 ± 0.13	1.67 ± 0.07	2.07 ± 0.09
'1' hr	1.99 ± 0.19	2.45 ± 0.14	1.82 ± 0.14	2.23 ± 0.17	2.37 ± 0.47	2.84 ± 0.49	1.59 ± 0.17	1.97 ± 0.09
'2' hr	2.27 ± 0.22	3.02 ± 0.08	2.32 ± 0.15	3.05 ± 0.14	2.44 ± 0.20	3.26 ± 0.19	2.01 ± 0.15	2.86 ± 0.12
'3' hr	1.70 ± 0.06	2.50 ± 0.19	1.68 ± 0.07	2.38 ± 0.16	2.14 ± 0.27	2.44 ± 0.29	2.56 ± 0.03	2.81 ± 0.04*

Experimental groups were compared with standard. $P < 0.05$.*$P < 0.01$

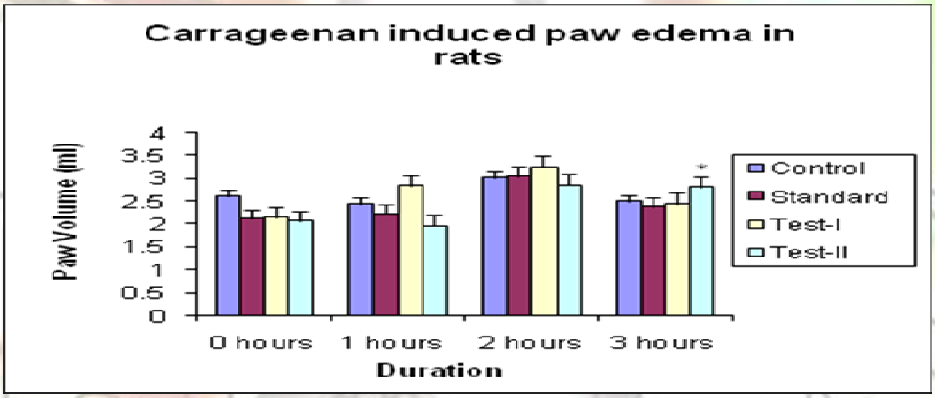

Fig.2 (A) Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using carrageenan induced paw edema in rats: Paw volume

Table 3 (B): Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using carrageenan induced paw edema in rats: Paw thickness

Time interval (hours)	Paw thickness (mm) Mean ± SEM (n=5)							
	Inflammation control		Inflammation standard		Inflammation Test-I		Inflammation Test-II	
	Control	Inflamed	Control	Inflamed	Control	Inflamed	Control	Inflamed
'0' hr	4.30 ± 0.12	10.4 ± 0.29	3.60 ± 0.18	9.10 ± 0.18	3.70 ± 0.25	8.80 ± 0.60	3.70 ± 0.25	8.80 ± 0.60
'1' hr	3.10 ± 0.18	8.20 ± 0.25	3.00 ± 0.15	7.80 ± 0.40	3.00 ± 0.22	8.30 ± 0.64	2.70 ± 0.25	7.90 ± 0.48
'2' hr	2.30 ± 0.12	7.20 ± 0.25	2.60 ± 0.10	6.90 ± 0.36	2.60 ± 0.10	7.30 ± 0.64	2.50 ± 0.15	6.90 ± 0.48
'3' hr	2.30 ± 0.12	6.20 ± 0.25	2.20 ± 0.12	5.90 ± 0.36	2.10 ± 0.10	6.20 ± 0.46	2.10 ± 0.10	6.00 ± 0.41

Experimental groups were compared with standard. $P < 0.05$.

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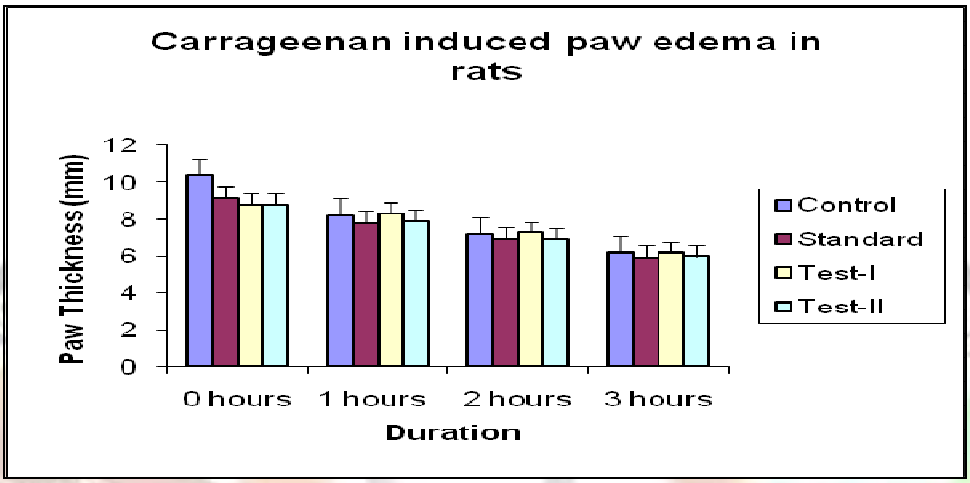

Leukocyte emigration in rats & other haematological parameters.

Table 4: Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using leukocyte emigration in rats & other haematological parameters

Groups & Treatment	Haematological parameters (Mean ± SEM) (n=5)			
	TLC (cells/cu.mm)	RBC(million/cu.mm)	Hb (gm%)	ESR (mm/hr)
Inflammation control	9300 ± 152.7	5.433± 0.185	7.5 ± 0.152	7 ± 0.577
Inflammation standard	7100 ± 208.1**	7.033 ± 0.120**	9.466 ± 0.120**	3.6 66 ± 0.333**
Inflammation test-I	7930 ± 35.11**	6.166 ± 0.145*	8.8 ± 0.173*	5.666 ±0.333
Inflammation test-II	7666.6 ± 88.1**	6.6 ± 0.152**	10.03 ± 0.463**	4.666 ± 0.333**

Acetic acid induced vascular (capillary) permeability method in rats.

Table 5: Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using acetic acid induced vascular (capillary) permeability method in rats.

Groups & Treatment	Dose (mg/kg)	Amount of dye Leakage(OD)	% inhibition
Inflammation Control	-	1.38 ± 0.035	
Inflammation Standard	10	0.835 ± 0.018**	39.5
Inflammation Test-I	100	1.078 ± 0.031**	21.9
Inflammation Test-II	200	1.020 ± 0.050*	26.1

Experimental groups were compared with control *P < 0.05 **P < 0.01

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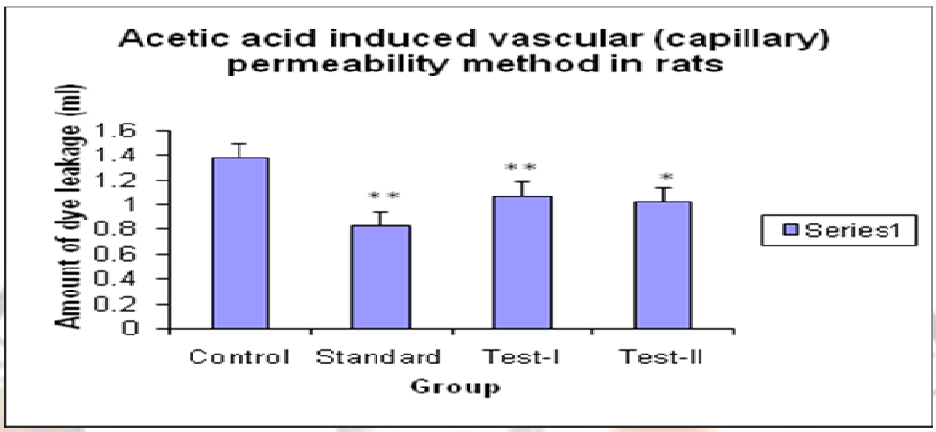

Fig. 3 Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using acetic acid induced vascular (capillary) permeability method in rats.

Mast cell degranulation in rats.

Table 6: Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using mast cell degranulation in rats.

Groups & Treatment	Dose (mg/kg)	% Inhibition of degranulation	Total mast cell.
Inflammation Control	-	15.2 ± 4.4	21.4 ± 3.6
Inflammation Standard	10	32.9 ± 4.6**	11.8 ± 1.3**
Inflammation Test-I	100	28.0 ± 7.6	4.40 ± 0.7***
Inflammation Test-II	200	35.0 ± 4.8**	0.70 ±0.2***

Experimental groups were compared with standard. *P < 0.05, **P < 0.01, ***P < 0.005

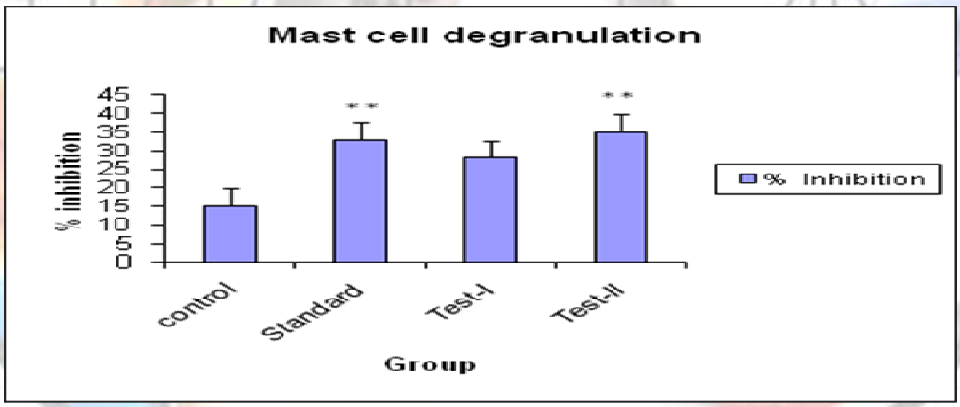

Fig. 4 (A) Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using mast cell degranulation in rats: % inhibition of mast cell degranulation.

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Fig. 4 (B) Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using mast cell degranulation in rats: Total mast cell.

Cotton pellet granuloma in mice

Table 7: Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using cotton pellet granuloma in mice

Cotton pellet granuloma in mice	Groups & Treatment			
	Inflammation Control	Inflammation Standard	Inflammation Test-I	Inflammation Test-II
Initial weight of cotton pellet	10.00 ± 0.00	10.00 ± 0.00	10.00 ± 0.00	10.00 ± 0.00
Dry granuloma weight	85.00 ± 2.51	68.67 ± 1.76*	62.33 ± 1.76*	66.00 ± 1.08*
% inhibition of granuloma	-	95.37 ± 2.45*	95.89 ± 2.71*	97.05 ± 1.58*

Values are expressed as Mean ± SEM.
(n = 5) Experimental groups were compared with standard. * P < 0.01

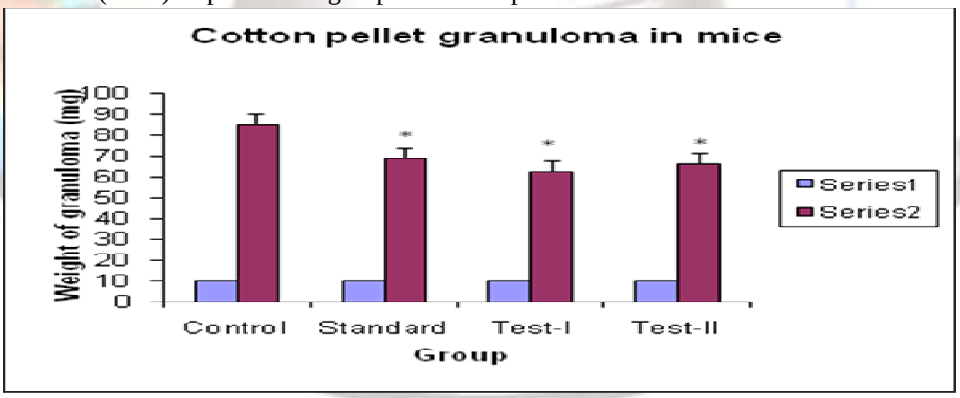

Fig. 5 Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using cotton pellet granuloma in mice.

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Formalin induced arthritis in rats.

Table 8 (A): Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using formalin induced arthritis in rats: Paw volume

Experimental groups were compared with standard. , P < 0.05, *P < 0.01.

Time interval (days)	Paw volume (ml) Mean ± SEM (n=5)							
	Formalin Arthritis control		Formalin Arthritis + standard		Formalin Arthritis + Test-I		Formalin Arthritis + Test-II	
	Control	Arthritic	Control	Arthritic	Control	Arthritic	Control	Arthritic
Day 1	1.90 ± 0.17	4.46 ± 0.08	2.02 ± 0.10	4.68 ± 0.22	2.06 ± 0.19	4.48 ± 0.30	2.18 ± 0.17	5.45 ± 0.35
Day 2	4.49 ± 0.53	8.39 ± 0.65	3.96 ± 0.46	8.89 ± 0.33	4.18 ± 0.21	8.85 ± 0.12	4.49 ± 0.62	8.23 ± 0.67
Day 3	4.36 ± 0.54	9.19 ± 0.37	4.04 ± 0.32	8.82 ± 0.35	4.37 ± 0.23	9.05 ± 0.24	5.01 ± 0.40	9.46 ± 0.28
Day 4	4.71 ± 0.57	9.69 ± 0.04*	4.03 ± 0.27	7.82 ± 0.35	4.21 ± 0.20	8.05 ± 0.24	4.64 ± 0.20	8.58 ± 0.09
Day 5	3.95 ± 0.32	8.69 ± 0.04*	3.30 ± 0.20	6.90 ± 0.60	3.37 ± 0.05	6.65 ± 0.13	3.39 ± 0.07	7.58 ± 0.09
Day 6	3.07 ± 0.20	7.69 ± 0.04*	2.88 ± 0.08	5.90 ± 0.60	2.49 ± 0.08	5.65 ± 0.13	2.39 ± 0.07	6.58 ± 0.09
Day 7	2.80 ± 0.18	7.28 ± 0.08	2.73 ± 0.03	5.35 ± 0.15	2.39 ± 0.08	5.37 ± 0.12	1.67 ± 0.42	4.41 ± 1.10
Day 8	2.27 ± 0.07	6.28 ± 0.08	2.25 ± 0.05	5.17 ± 0.07	2.12 ± 0.02	4.37 ± 0.12	1.60 ± 0.40	3.61 ± 0.90

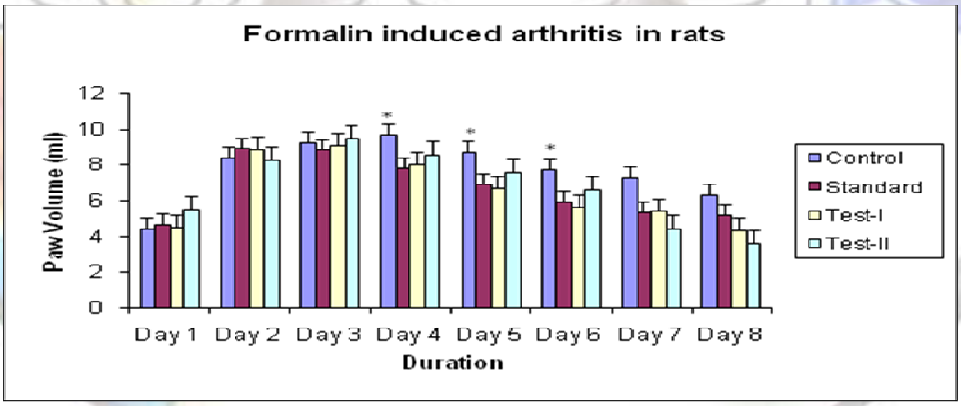

Fig 6 (A) : Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using formalin induced arthritis in rats: Paw volume

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Table 8(B): Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using formalin induced arthritis in rats: Paw thickness

Experimental groups were compared with standard.P < 0.05

Time interval (days)	Paw thickness (mm) Mean ± SEM (n=5)							
	Formalin Arthritis control		Formalin Arthritis + standard		Formalin Arthritis + Test-I		Formalin Arthritis + Test-II	
	Control	Arthritic	Control	Arthritic	Control	Arthritic	Control	Arthritic
Day 1	3.00 ± 0.22	6.80 ± 0.25	3.30 ± 0.25	7.10 ± 0.43	3.20 ± 0.25	7.00 ± 0.35	3.10 ± 0.29	6.90 ± 0.36
Day 2	3.00 ± 0.22	6.80 ± 0.25	3.30 ± 0.25	7.10 ± 0.43	3.20 ± 0.25	7.00 ± 0.35	2.75 ± 0.14	7.00 ± 0.54
Day 3	3.20 ± 0.20	7.40 ± 0.33	3.66 ± 0.44	8.00 ± 0.28	3.30 ± 0.25	7.00 ± 0.35	2.87 ± 0.31	7.12 ± 0.55
Day 4	3.75 ± 0.32	9.25 ± 0.32	3.50 ± 0.28	8.83 ± 0.44	3.70 ± 0.25	8.80 ± 0.60	3.25 ± 0.32	8.75 ± 0.59
Day 5	2.75 ± 0.32	8.25 ± 0.32	3.00 ± 0.00	7.75 ± 0.75	2.70 ± 0.25	7.80 ± 0.60	2.37 ± 0.23	7.75 ± 0.59
Day 6	2.75 ± 0.25	7.00 ± 0.35	2.25 ± 0.25	6.75 ± 0.75	2.40 ± 0.10	6.80 ± 0.46	2.25 ± 0.14	6.37 ± 0.42
Day 7	2.25 ± 0.14	6.50 ± 0.35	2.50 ± 0.00	6.25 ± 0.25	2.40 ± 0.10	6.20 ± 0.25	2.25 ± 0.14	5.62 ± 0.31
Day 8	2.25 ± 0.14	5.87 ± 0.23	2.50 ± 0.00	5.00 ± 0.50	2.40 ± 0.10	5.50 ± 0.22	2.25 ± 0.14	4.62 ± 0.31

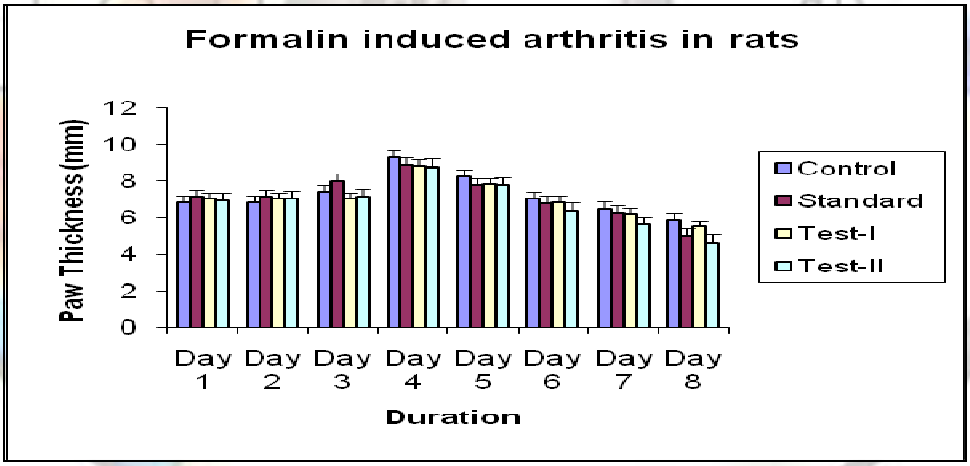

Fig 6 (B) Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using formalin induced arthritis in rats: Paw thickness

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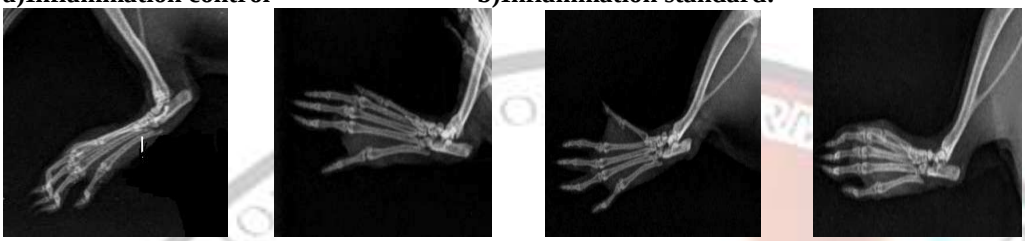
Radiographic analysis in formalin induced arthritis in rats

a)Inflammation control **b)Inflammation standard:**



c) Inflammation Test-I **d)Inflammation Test-II**

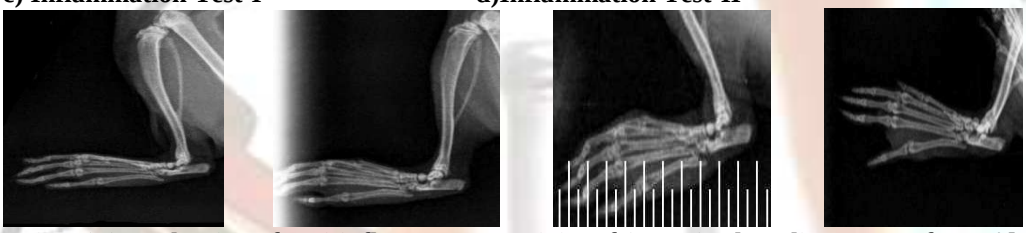
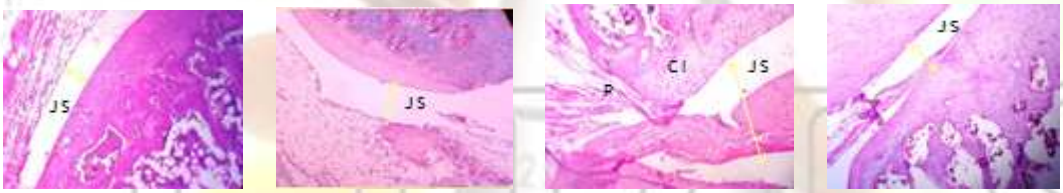


Fig 6 (C) Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using formalin induced arthritis in rats: Radiographic analysis

Histopathological changes in formalin induced arthritis in rats.

Inflammation Control **Inflammation Standard**



Inflammation Test-I **Inflammation Test-II**

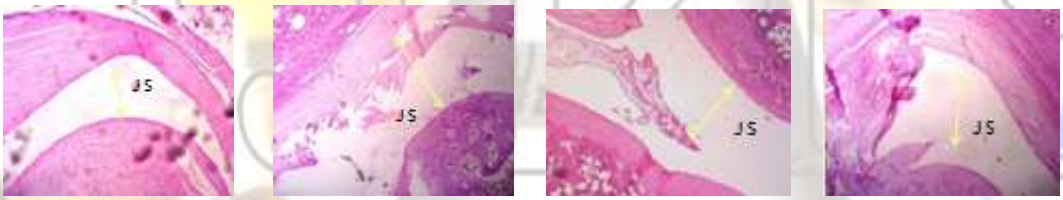


Fig 6 (D) Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using formalin induced arthritis in rats: Histopathological changes.

Delayed type hypersensitivity in rats

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Table 9 (A): Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using delayed type hypersensitivity in rats: Paw volume

Experimental groups were compared with standard. * P < 0.05 **P < 0.01.

Time interval (hours)	Paw volume (ml) Mean ± SEM (n=5)							
	DTH control		DTH + standard		DTH + Test-I		DTH+ Test-II	
	Control	Inflamed	Control	Inflamed	Control	Inflamed	Control	Inflamed
'0' hr	2.31 ± 0.11	3.63 ± 0.15*	2.48 ± 0.11	3.29 ± 0.15*	2.64 ± 0.03	3.68 ± 0.09*	2.82 ± 0.06	3.84 ± 0.10*
'48' hr	1.42 ± 0.10	2.40 ± 0.11*	1.39 ± 0.05	2.29 ± 0.03**	1.36 ± 0.04	3.68 ± 0.10*	1.23 ± 0.05	2.17 ± 0.05*

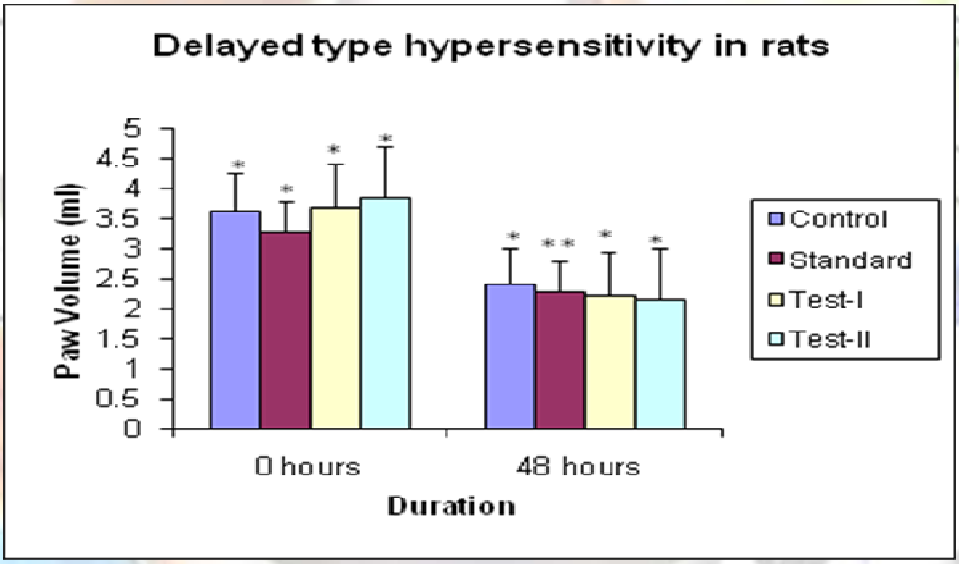

Fig 7 (A) Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using delayed type hypersensitivity in rats: Paw volume.

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Table 9 (B): Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using delayed type hypersensitivity in rats: Paw thickness.

Experimental groups were compared with standard. $P < 0.05$.

Time interval (hours)	Paw thickness (mm) Mean ± SEM (n=5)							
	DTH control		DTH + standard		DTH + Test-I		DTH+ Test-II	
	Control	Inflamed	Control	Inflamed	Control	Inflamed	Control	Inflamed
'0' hr	3.30 ± 0.12	9.40 ± 0.29	2.80 ± 0.12	8.10 ± 0.18	3.10 ± 0.18	7.80 ± 0.60	2.82 ± 0.06	3.84 ± 0.10
'48' hr	2.60 ± 0.10	9.40 ± 0.30	2.40 ± 0.10	6.00 ± 0.35	2.60 ± 0.10	6.60 ± 0.33	1.23 ± 0.05	2.17 ± 0.05

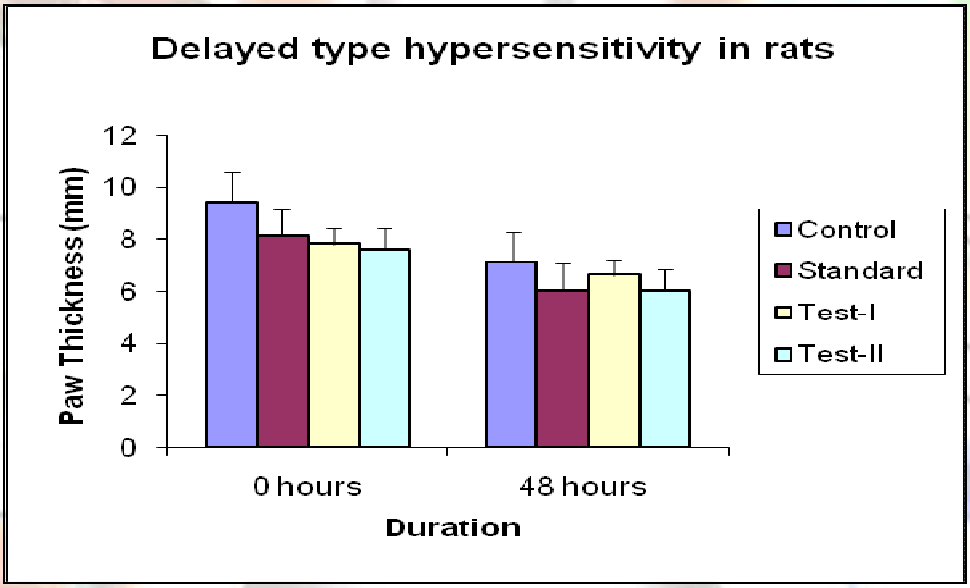

Fig 7 (B) Evaluation of anti-inflammatory activity of (EEHI) ethanolic extract of *Hemidesmus indicus* using delayed type hypersensitivity in rats: Paw thickness.