



Research Article

Euphorbia prostrata Exerts Potent Anti-Inflammatory and Anti-Arthritic Activity in Downregulating the Increased Expression of Pro-Inflammatory Cytokines

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Abstract

Background: *Euphorbia prostrata* constitutes a herbal medication widely used to cure numerous inflammatory diseases occurring either alone or in conjunction with other herbal formulations. The research conducted was devised with the aim of determining the effect of *Euphorbia prostrata* hydroalcoholic leaf extract on paw swelling, joint destruction, and the formation of inflammation-producing cytokines in animal models of rheumatoid arthritis.

Methods: Hydroalcoholic *Euphorbia prostrata* extract and a reference drug (indomethacin 3 mg/kg), were both administered orally on a daily basis at varying doses; low (50 mg/kg), medium (100 mg/kg), and high (200 mg/kg) for a period of 21 days. Other parameters affecting the functional components of bone include joint diameter measurements and histopathological investigations. Immunohistochemical analysis of Interleukin (IL-1, IL-6) and Nuclear Factor (NF-κB) in ankle joint tissue was performed.

Results: The research findings indicated that a significant ($p < 0.05$) dose-dependent reduction in inflammation results from the administering of *Euphorbia prostrata* at varying doses. A 200 mg/kg dose of *Euphorbia prostrata* with a significance of $p < 0.001$ produced a marked reduction in both inflammation and joint dysfunction. It was concluded, therefore, that such a dose attenuates paw oedema and inflammation, while also reversing bone damage through the inhibition of activated pro-inflammatory mediators and, specifically, NF-κB-mediated production of cytokines.

Conclusion: The research presented here concludes that *Euphorbia prostrata* hydroalcoholic extract can be potentially employed in the treatment and management of rheumatoid arthritis since it reduces symptoms of inflammation, inhibits macrophage activity and modulates IL-1, IL-6 and NF-κB.

Introduction

Inflammatory disorders such as rheumatoid arthritis (RA) affect a significant proportion of population. In 2015, the prevalence rate of RA in India was found to be approximately 0.75% of the total adult population.^{1,2} The disease is characterized as a persistent autoimmune-mediated inflammatory disorder with infiltration of cellular components and subsequent proliferation of synovial cells. It can also induce the formation of pannus, destruction of cartilage and erosion of joints, the last-mentioned being due to the presence of infiltrating cells, other cytokines, proteolytic enzymes and prostanoids.³ The disease pathogenesis is initiated due to the production of free radical species and presence of pro-inflammatory cytokines at the inflammation site.⁴ A variety of mechanistic pathways is implicated in the emergence

of RA, some involving dysregulation or imbalance in anti-inflammatory and pro-inflammatory cytokines. The accumulation of macrophages and neutrophils, together with the production of free radical-producing enzymes in synovial fluid are alleged to constitute the initial stage of RA. High levels of reactive oxygen species (ROS) in the synovial cavity stimulate the inflammatory process by recruiting NF-κB and inducing the release of other numerous transcription mediators, cytokines and neutrophils.⁵ Several pharmacological agents such as glucocorticoids and methotrexate have been shown to inhibit pro-inflammatory cytokine production and, thus, attenuate joint destruction.^{6,7} Consequently, there are numerous plant-based phytoconstituents that possess significant therapeutic value (such as the inhibition of

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NF- κ B and pro-inflammatory cytokines) and are potential leading candidates as remedies for RA.⁸ Therefore, identification of natural substances that protect the tissues from chronic inflammatory disorders would offer new opportunities to enhance RA therapy.

Euphorbia prostrata Linn. is a perennial herb, growing in abundance throughout Africa and India, which has traditionally been used for the treatment of diarrhea, asthma, allergies, hemorrhoids, and diabetes mellitus.⁹⁻¹² The active chemical constituents of *Euphorbia prostrata* are phenolic acid, flavonoids and tannins.¹³ Various reports exist indicating that flavonoids and phenolic acid possess analgesic, anti-inflammatory, anti-thrombotic, antioxidant, haemostatic and vasoprotective qualities.¹⁴⁻¹⁶ Chemical analysis of *Euphorbia prostrata* has proven that its phenolic compounds execute specific roles. For example, gallic acid activates the Hageman factor and is responsible for hypercoagulability. Ellagic acid suppresses histamine release, while flavonoids such as apigenin both inhibit I κ -B and suppress inflammatory mediators and luteolin inhibits protein tyrosin phosphorylation. Tannins possess haemostatic and astringent properties and numerous preclinical studies have confirmed their promoting of wound healing and anti-hemorrhoidal activity. Several studies have reported the efficacy of standardized extracts of *Euphorbia prostrata* in treating carrageenan-induced paw oedema and histamine-induced oedema.¹⁷ Moreover, they represent a potential cure for various ailments. This academic article represents the first study to date of the mechanism underpinning the attenuating role of hydroalcoholic extract of *Euphorbia prostrata* leaves in inflammation and bone damage. Therefore, the current study was intended to examine the potential effect of hydroalcoholic *Euphorbia prostrata* extract (HEPE) on inflammation and bone damage in animal research models of inflammation and RA via modulating IL-1, IL-6, and NF- κ B pathways.

Materials and Methods

Animal study

The research conducted involved the use of 5-7 week old albino Wistar rats, weighing 170-200 grams. The investigative procedure was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) which issued protocol approval number 760/IAEC/13 covering all the experiments conducted. The investigative methods implemented adhered strictly to the Indian National Science Academy Guidelines for Care and Use of Animals in Scientific Research and the Code of Ethics of the World Medical Association (Declaration of Helsinki). EU Directive 2010/63/EU for animal-based experiments. The animal research subjects were quarantined in standard laboratories at a temperature of $25 \pm 2^\circ\text{C}$ and a relative humidity of $55 \pm 5\%$ following a 12-hour light/dark cycle. They were placed on dry paddy husks with unrestricted access to food and water.

Chemicals and reagents

For the purposes of inducing arthritis in the research subjects, Complete Freund's adjuvant (CFA) was procured from Difco Laboratories Inc., USA. The reference drug, Indomethacin, was obtained from Sigma Chemical Co., USA. The immunohistochemistry kit was purchased from Vector labs, CA, USA. Primary antibodies of IL-1, IL-6, NF- κ B were procured from Santa Cruz, CA, USA. All the other chemicals employed were of analytical grade.

Investigational drug

Fresh Leaves of the *Euphorbia prostrata* were sourced in the local market and validated by a botanist from the Department of Pharmacognosy and Phytochemistry, Jamia Hamdard, New Delhi. Voucher specimens No. (Pharma/008/2013) were duly placed in the department herbarium.

Extraction procedure

The extraction procedure involved the use of *Euphorbia prostrata* leaves which were macerated for seven days in a 50:50 mixture of distilled water and methanol while being regularly agitated. The supernatant was subsequently transferred and filtered and the solvent evaporated in a rotary evaporator. The yield was calculated and the physical characteristics of the crude extract established through reference to the dried weight of *Euphorbia prostrata* leaves.

Quantitative tests for *Euphorbia prostrata* (HEPE)

The quantitative determination of gallic and ellagic acid in HEPE was established by means of HPTLC analysis. Hydroalcoholic extracts of *Euphorbia prostrata* leaves were marked on precoated 0.2 mm thick silica gel plates 20 x 10 cms in size with a Camag microlitre syringe and a Camag linomat 3 applicator. An automatic sample spotter was employed before the plates were subsequently developed in an appropriate solvent system, namely; Toluene: ethyl acetate: formic acid (4:5:2) v/v in a Camag glass twin trough chamber (pre-saturated with a solvent for 30 minutes). All the precoated silica gel plates were air-dried prior to scanning with a Camag TLC scanner at 254 nm absorbance employing Wincats version 4.03 software. Photodensitograms of the fluorescent HPTLC plate of the samples depicted by TLC scanner CAMAG are represented in Figure 1.

Experimental methodology

Toxicity studies of HEPE

Assessment of the acute oral toxicity of plant extract was completed in accordance with the Organization for Economic Co-operation and Development (OECD-423) guidelines for the testing of chemicals.¹⁸ Initially, a limit test involving the administering of a 5000 mg/kg dose was conducted on five male and five female Wistar rats whose weight fell within the mean range of 180-250 grams. Consequently, the experimental subjects were segregated into two groups each containing six members.

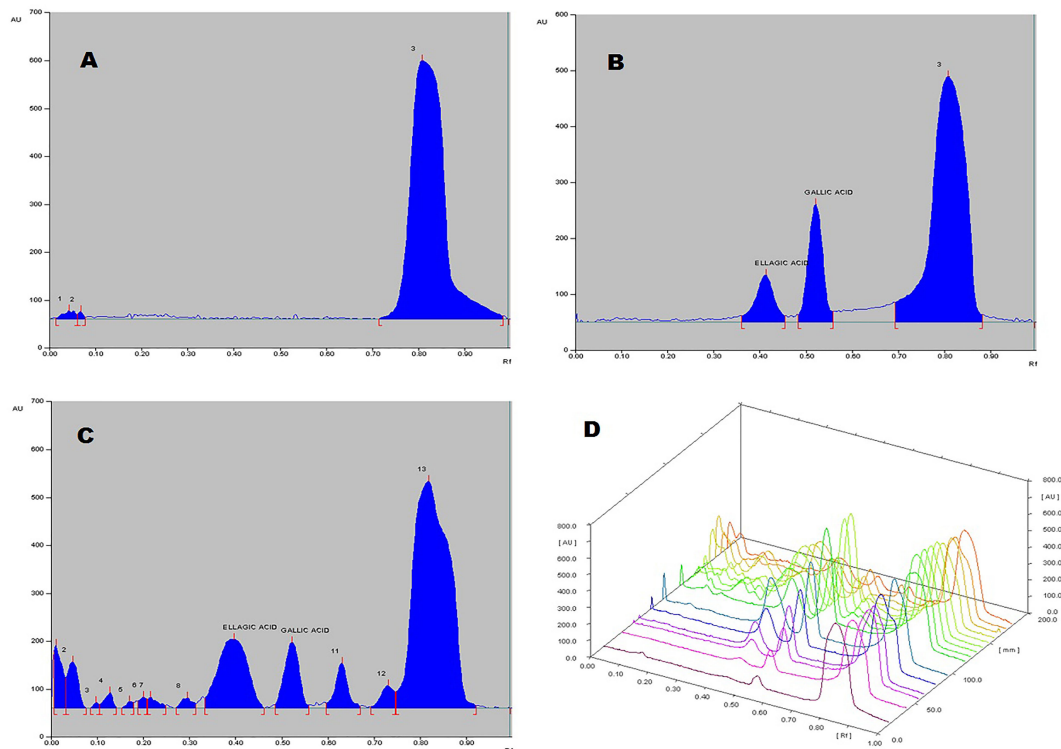


Figure 1. Photodensitogram of the fluorescent HPTLC plate of the samples depicted by TLC scanner CAMAG of Gallic and Ellagic acid in HEPE (A). Blank spectrum (B). Standard (Gallic and Ellagic acid) spectrum (C). *Euphorbia prostrata* extract (D). Axis represent, x= Retention factor and y= absorption

After administration of the test dose, the subjects were observed during a 14-day study period in order to detect any changes in behavior or signs of mortality. The 28-day sub-acute repeated toxicity study was carried out in accordance with OECD guidelines for the testing of chemicals (OECD-407)¹⁹ using the following subject groupings:

- Group 1, as the normal control, received the vehicle (0.5% Carboxymethyl Cellulose (CMC) at 2 ml/kg body weight
- Group II received plant extract at 200 mg/kg body weight

The experimental study subjects consisted of Wistar rats of both genders, 150-180 grams in weight, drawn from the breeding stock of the researchers. The plant extract and vehicle were administered orally to the respective groups on a daily basis during a study period of 28 days.²⁰ At that point in time, the subjects were sacrificed to enable study of toxicity in their livers, kidneys, hearts, brains, testes and ovaries.

Formaldehyde-induced arthritis

Female albino Wistar rats were segregated into five groups with N=6 subjects per group. The joint diameter (baseline value) was measured by means of a micrometer screw gauge. Plant extract and vehicle were administered daily to the respective groups during a study period of 10 days. On day 1, induction of arthritis was achieved by injecting 0.1 ml of formaldehyde at a concentration of 2 % v/v into the

sub plantar surface 30 minutes after the administration of plant extract and vehicle. The 0.1 ml of formaldehyde was injected via the subplantar route into the left hind paws of all subjects on days 1 and 3. Changes in the paw diameter values in relation to the baseline values were subsequently measured on days 8, 9 and 10.²⁰

Carrageenan-induced paw oedema

Wistar rats were segregated into five groups with N=6 subjects/group. The experimental subjects were obliged to fast overnight, although they had free access to drinking water. The paw volume corresponding to the volume displaced (conductivity solution) was measured using a plethysmometer (Ugo Basile, USA). The baseline values were recorded on day 0 with the treatment and vehicle administration subsequently continuing on day 1. The experimental designs adopted within this methodology were as follows:

Group 1: served as the normal control and received the vehicle (2 ml/kg body wt).

Group II: served as the reference drug and received Indomethacin (3 mg/kg body wt).

Groups III-V: served as the treatment groups and received plant extract in three doses of mg/kg body wt.

The plant extract, vehicle and reference drug were administered orally on day 1. Thirty minutes later, oedema was induced in the experimental subjects. The induction of anti-inflammatory activity was effected by injecting carrageenan at 1% concentration into their hind paws.

0.1 ml of carrageenan was injected into the subplantar surface of their left hind paws. The change in paw volume was subsequently measured using the following plethysmometer instrument scale: 0 (baseline), 1, 3 and 6 hours. The % inhibition in oedema was calculated using the equation below:

Percentage oedema inhibition = $(V_{\text{control}} - V_{\text{treatment}} / V_{\text{control}}) \times 100$
 where, $V_{\text{treatment}}$ represents the mean increase in paw volume in the treatment group of subjects and V_{control} represents the mean increase in paw volume of control group members.^{21,22}

Cotton pellet granuloma test

Wistar rat subjects were segregated into five groups with N=6 subjects per group and the experimental methodology adopted matched that of the previous experiment, i.e. carrageenan-induced paw oedema. Prior to initiating the experiment, the subjects were denied food overnight. Administration of plant extracts, vehicles and the reference drug to the respective subject groups was either by oral means or gastric gavage. After the elapse of thirty minutes, the subjects were anaesthetized with ether. Thereafter, granuloma was induced by the bilateral implanting of a sterile cotton pellet, weighing approximately 30 ± 1 mg that had been saturated with isotonic saline solution beneath the epidermis and axilla. The subjects were maintained in an aseptic environment during the study period. The oral administration of plant extracts, reference drug and vehicle was continued for seven days with all subjects being sacrificed on day 8. The cotton pellets previously embedded in their pouches were excised and dehydrated in an oven at 60°C. The weight of the pellets after drying was measured three times until a constant weight was recorded. The difference between the initial and final cotton pellet weights were calculated and considered the dry weight of the granuloma tissue.^{23,24}

Adjuvant-induced arthritis

Albino Wistar rats were segregated into five groups with N=6 subjects per group. The grouping of subjects was undertaken in accordance with the experimental design employed in the carrageenan-induced paw oedema model. Joint diameters were measured with a micrometer screw gauge on day 0 and recorded as baseline values. Plant extract, reference drugs and vehicles were administered to their respective subjects after the baseline recording. Thirty minutes later, induction of arthritis was performed by injecting 0.1 ml of CFA (adjuvant containing 0.05% w/v *M. butyricum* dissolved in mineral oil) into the sub plantar surface of the left hind paw of all subjects. Immunization with CFA in the left hind paw was performed on day 1 with oral administration of plant extracts/vehicle being continued for 20 days. Meanwhile, measurement of the left hind paw diameter was completed on days 3, 7, 14 and 21. The subjects were evaluated on a daily basis for the presence of arthritis. On day 21, they were sacrificed and their left hind paws stored in formalin for later

histopathological evaluation at -20°C for the purposes of immunohistochemical assay and radiographic analysis.^{20,24}

Histopathological investigation

Histology of the left ankle joints was undertaken by embedding them in 10% formaldehyde solution. Thereafter, decalcification in 10% Ethylenediaminetetraacetic acid (EDTA) for a period of 24 hours was completed. The softening of bony tissue was achieved by decalcification, while dehydration involved the use of ethanol before it was embedded in paraffin wax. 5 µm sections were cut, stained with a combination of haematoxylin and eosin (H&E) and observed through an optical microscope. Changes in the anatomy of the ankle joint sections of all the subjects or inflammation of these sections were recorded.

Radiographic investigation

A radiographical examination of the hind paw ankle joints of subjects was performed on the 21st day in order to quantify soft tissue swelling, bone erosion and the destruction of cartilage. All subjects were administered intravenous anesthetics before undergoing a radiographic X-ray with the distance between the machine and the source being maintained at 90 cm (Philips X12, Germany). Radiographic scoring of the control and treatment groups was conducted on a scale of 0-3. Scale 0 indicated no damage, while scales 1, 2, 3 signified mild, moderate and severe damage respectively.²⁵

Immunohistochemical analysis

The expression of various pro-inflammatory IL-1β, IL-6, and NF-κB was examined immunohistochemically. The decalcified ankle joints were sectioned using a cryotome. Thin frozen sections (6 µm) were produced using a cryotome and after fixation with acetone, the corresponding sections were processed to form slides which were then treated with 30% H₂O₂ in methanol to arrest any endogenous peroxidase activity present in ankle joint tissues. The slides were blocked with bovine serum albumin at RT for a period of one hour followed by incubation with primary monoclonal antibodies against IL-1β (1:200), IL-6 (1:200), NF-κB (1:200) for 48 hours. After treatment with primary antibodies, 2-hour incubation with Horse Radish Peroxidase (HRP)-conjugated secondary antibody (1:2000) (Santa Cruz, CA, USA) was completed. Treatment with 3,3'-diaminobenzidine (DAB) through the use of a DAB substrate kit (Vector Labs, CA, USA) produced a colorogenic immune reaction. The slides were then mounted with Distrene-Plasticizer-Xylol (DPX) to enable observation of IL-1β, IL-6, and NF-κB activity under an optical microscope (Nikon ECLIPSE E600, Japan).

Statistical analysis

Data was reported in the form of Mean ± SEM (standard error of the mean). Statistical analysis was undertaken by means of ANOVA (One-way analysis of variance) with a Dunnett's multiple comparison post hoc test used to

calculate significance. All the statistical analysis involved calculations performed with a Graph pad Prism version 5.03, (San Diego, CA, USA), where $p < 0.05$ was taken to be statistically significant.

Results

Extraction and standardization of hydroalcoholic extract of *Euphorbia prostrata* (HEPE)

The yield of HEPE was found to be 20.19% and the extract appeared greenish brown in color. The quantitative estimation of *Euphorbia prostrata* by HPTLC revealed the presence of gallic acid at 23.83 mg/kg and ellagic acid at 139.3 mg/kg and was calculated on the basis of the following regression equation:

$Y = 4.582X + 2975$ ($R^2 = 0.993$) Regression equation of gallic acid

$Y = 5.441X + 1035$ ($R^2 = 0.997$) Regression equation of ellagic acid

Toxicity study of HEPE

In an acute toxicity study, the administration of HEPE at a dose of 5000mg/kg body weight culminated in the survival of all test group subjects (Table 1).

Therefore, the results demonstrated that HEPE can be considered safe in rats at a dose of 5000 mg/kg. In a subacute toxicity study, the subjects survived through chronic administration of HEPE (200 mg/kg) for 28 days. No

Table 1. Effect of administering HEPE on the mortality rate in a 14-day acute oral toxicity study.

Treatment Group	No. of subjects alive (M/F)
Normal Control	5/5
<i>Euphorbia prostrata</i> treated	5/5

Table 2. Effect of control and HEPE on organ weight (liver, kidney and testis) during a 28-day subacute toxicity study. All values are mean \pm SEM (n=6). Statistical analysis by paired t-test.

Drug Treatment	Organ Weight (gm)					
	Liver	Kidney	Heart	Brain	Ovary	Testis
Control (female)	6.73 \pm 0.08	1.28 \pm 0.02	0.67 \pm 0.05	1.91 \pm 0.02	0.073 \pm 0.01	-
Control (male)	8.16 \pm 0.06	1.79 \pm 0.01	0.88 \pm 0.017	2.06 \pm 0.02	-	1.10
HEPE (female 200mg/kg)	6.36 \pm 0.2	1.33 \pm 0.04	0.68 \pm 0.04	1.88 \pm 0.02	0.076 \pm 0.01	-
HEPE (male 200mg/kg)	8.00 \pm 0.10	1.74 \pm 0.02	0.83 \pm 0.02	2.04 \pm 0.03	-	1.07

Table 3. Effect of control and HEPE on biochemical parameters during a 28-day subacute toxicity study. All values are mean \pm SEM (n=6). Statistical analysis by paired t-test.

Drug Treatment	Biochemical Parameters					
	Blood glucose level (mg/dl)	Triglycerides level (mg/dl)	HDL level (mg/dl)	Serum creatinine level (mg/dl)	Alanine transferase level (U/L)	Aspartate amino transferase level (U/L)
Control	116 \pm 2.4	52.75 \pm 2.6	34.54 \pm 1.4	0.74 \pm 0.04	42.60 \pm 1.6	105.60 \pm 3.7
HEPE (200mg/kg)	111 \pm 4.7	55.74 \pm 2.3	39.68 \pm 1.1	0.75 \pm 0.03	45.50 \pm 2.00	106.30 \pm 5.9

Table 4. Effect of control and HEPE on haematological parameters during 28-day subacute toxicity study. All values are mean \pm SEM (n=6). Statistical analysis by paired t-test.

Drug Treatment	Haematological Parameters					
	RBC (million/mm ³)	WBC (thousand/mm ³)	Platelet (lacs/mm ³)	Haemoglobin (g/dl)	Bleeding time (sec)	Clotting time (sec)
Control	7.69 \pm 0.08	17.93 \pm 0.3	37.19 \pm 2.0	13.38 \pm 0.6	834 \pm 13.30	231.50 \pm 3.7
HEPE (200mg/kg)	7.30 \pm 0.2	17.68 \pm 0.3	39.85 \pm 2.0	13.62 \pm 0.61	844 \pm 14.13	227.8 \pm 3.7

significant changes were observed in body weight (Figure 2), organ weight (Table 2), biochemical parameters (Table 3) or haematological parameters (Table 4) in comparison to the control. No histopathological changes (Figure 3) were observed in the test subjects.

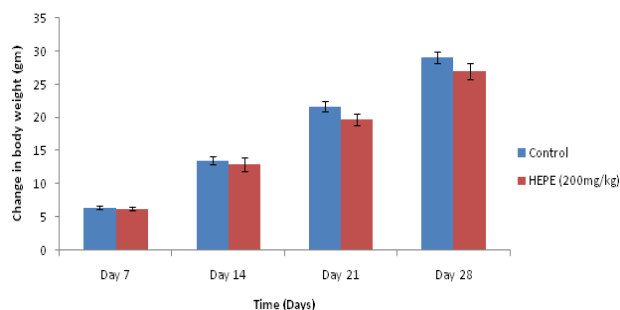


Figure 2. Effect of control and HEPE (200 mg/kg) on changes in body weight on days 7, 14, 21 and 28 during 28-day subacute toxicity study. All values are expressed as Mean \pm SEM (n=6). Statistical analysis by paired t-test.

Effect of HEPE on formaldehyde-induced arthritis

The formaldehyde at a concentration of 2% v/v in experimental subjects caused prominent and pronounced swelling of the left ankle joints on days 1 and 3 for a period of 10 days. The change in joint diameter was found to be less pronounced in groups treated with Indomethacin and hydroalcoholic *Euphorbia prostrata* extract (HEPE) in comparison to the control. This variation was significant ($p < 0.01$) in groups administered with HEPE 100mg/kg and HEPE 200 mg/kg during the entire study period. Even though HEPE caused a dose dependent inhibition of joint swelling, maximum inhibition was produced by Indomethacin (Figure 4).

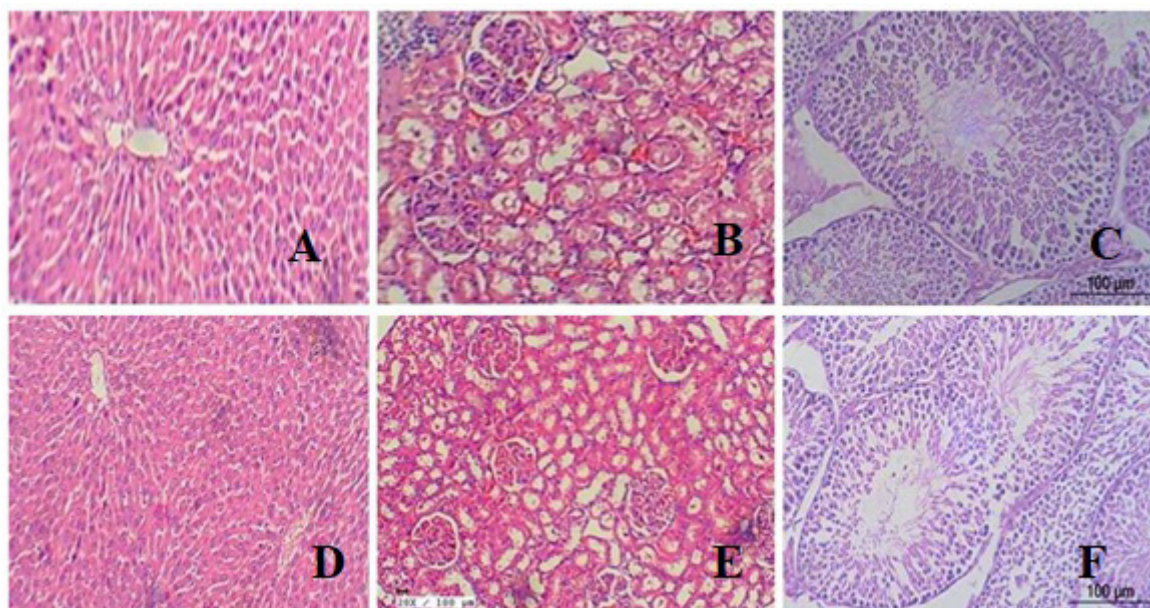


Figure 3. Effect of control and HEPE (200 mg/kg) on histopathology of various organs (liver, kidney and testis) during 28-day subacute toxicity study. Hematoxylin & Eosin stained section pictomicrographs were taken at 20x magnification. Pictomicrographs demonstrate no significant histopathological changes after administration of HEPE extract as compared to the control sections. A. Control (Liver) B. Control (Kidney) C. Control (Testis) D. HEPE treated (Liver) E. HEPE treated (Kidney) F. HEPE treated (Testis).

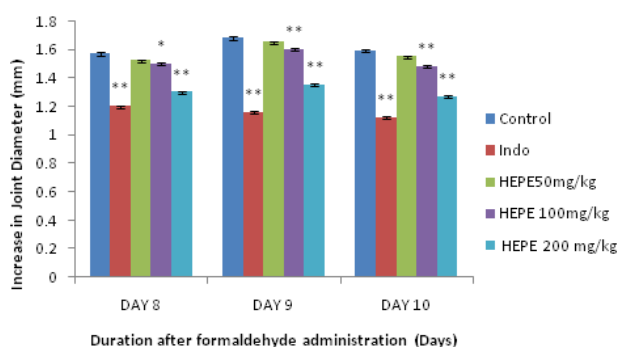


Figure 4. Effect of 50, 100 and 200 mg/kg doses of formaldehyde control, Indomethacin and HEPE on joint diameter increase resulting from formaldehyde-induced arthritis on days 8, 9 and 10. Statistical analysis was carried out by one-way ANOVA with a Dunnett's multiple comparison test.

Effect of HEPE on carrageenan-induced paw oedema

Carrageenan injected into the sub plantar surface caused an increase in paw oedema which persisted during the observation period. After carrageenan injection, maximum paw swelling was observed after six hours. The reference drug, Indomethacin, caused a significant reduction in the paw diameter. HEPE treatment produced a maximum reduction in paw oedema at the two doses of 100 and 200 mg/kg, 1 hour, 3 hours and 6 hours after carrageenan injection. However the decrease in paw oedema was statistically significant ($p < 0.01$) at 200mg/kg dose (Figure 5).

Effect of HEPE on cotton pellet granuloma test

The subcutaneous implanting of cotton pellets in the axillary region of the experimental subjects led to the formation of granulomatous tissue accompanied by foreign material. In both the reference and HEPE-treated groups, a

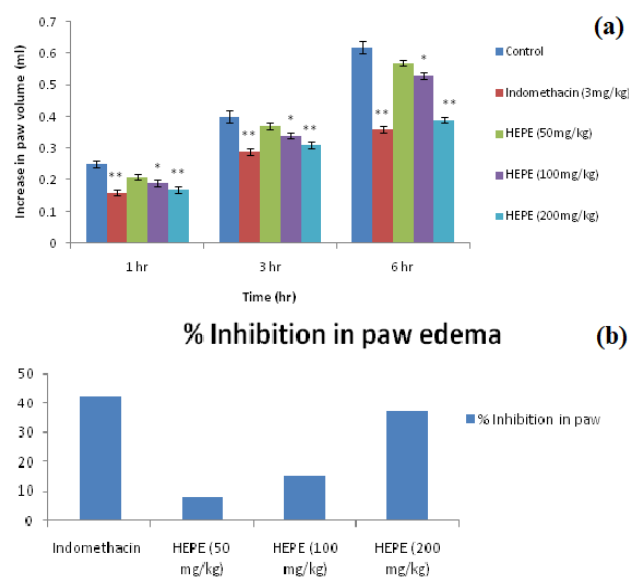


Figure 5. (a). Effect of 50, 100 and 200 mg/kg doses of carrageenan control, Indomethacin and HEPE on the increase in paw volume in carrageenan-induced paw oedema at 1, 3, and 6 hours. All values are Mean±SEM (n=6). Statistical analysis by one way ANOVA followed by Dunnett's Multiple Comparison. * $p < 0.05$, ** $p < 0.01$. **(b).** Effect of 50, 100 and 200 mg/kg doses of Indomethacin and HEPE on the percentage inhibition of paw oedema in a carrageenan-induced model. All values are Mean±SEM (n=6).

significant ($p < 0.05$) decrease in the dry granuloma weight of sterile cotton pellet was noted. The treatment with HEPE produced a dose-dependent diminution in granuloma weight (Figure 6) and this decrease was significant at two doses, 100 and 200 mg/kg.

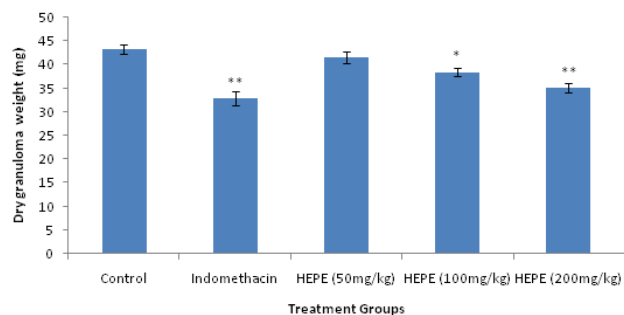


Figure 6. Effect of 50, 100 & 200 mg/kg doses of control, Indomethacin and HEPE on dry granuloma weight (mg) in cotton pellet-induced granuloma models. All values are Mean±SEM (n=6). Statistical analysis by one way ANOVA followed by Dunnett's Multiple Comparison. *p<0.05, **p<0.01.

Effect of HEPE on adjuvant-induced arthritis

CFA administration-induced arthritis augmented the joint diameter. The largest increase in joint diameter was observed on day 3 in the treatment group members. A gradual decrease in joint diameter was observed during the study period of 21 days, except in the CFA-control group. In the CFA-group, an increase in joint diameter was noted between days 14 and 21 (Figure 7). However, the reduction in joint swelling was statistically significant at a dose of 200 mg/kg. Consequently, this dose was subjected to further histopathological, radiographical and immunohistochemical analysis.

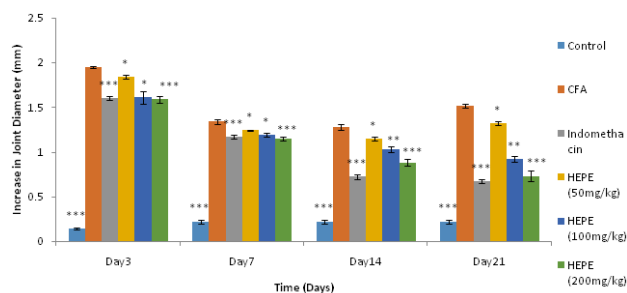


Figure 7. Effect of 50, 100 & 200 mg/kg doses of CFA-control, Indomethacin and HEPE on increases in joint diameter in a model of CFA-induced arthritis on days 3, 7, 14 and 21. All values are Mean±SEM (n=6). Statistical analysis by one way ANOVA followed by Dunnett's Multiple Comparison. *p<0.05, **p<0.01.

Histopathological assessment

Noteworthy morphological changes in the joints of CFA-induced subjects (Figure 8A1) were observed in comparison to those resulting from administration of the standard drug Indomethacin (Figure 8B1). Pictographic representations depict the ankle joints of CFA-induced subjects as being inflamed and oedematous. The group administered with HEPE exhibited significant protection from CFA-induced arthritis-related joint changes (Figure 8B3). The result of histopathological examination of ankle joint tissue of Indomethacin (3 mg/kg) group showed that the synovial space, lining of synovial and articular cartilage were all normal and no significant pathology was evident (Figure 8B2). In contrast, in the ankle joints of arthritic

subjects the synovial space was decreased and the synovial membrane was both thickened and oedematous. Articular cartilages were damaged and blood vessels dilated (Figure 8A2). The group administered with HEPE demonstrated decreased synovial hyperplasia and inflammatory cell infiltration when compared to the control (saline-treated arthritic) group subjects (Figure 8C2).

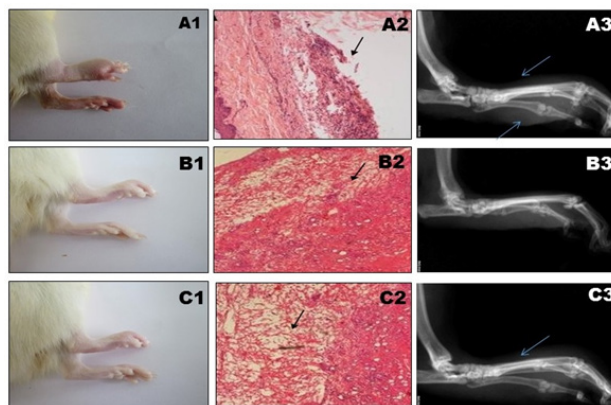


Figure 8. Effect of 200 mg/kg doses of CFA-control, Indomethacin and HEPE on morphological, histopathological and radiographical changes in CFA-induced arthritis. Morphology of knee joint of CFA-control (A1), Indomethacin (B1) and HEPE at 200 mg/kg (C1) Histopathology of knee joint of CFA-control (A2), Indomethacin (B2) and HEPE at 200 mg/kg (C2) Radiology of knee joint of CFA-control (A3), Indomethacin (B3) and HEPE at 200 mg/kg (C3).

Radiographic assessment

Figure 8 (A3-C3) contains X-ray radiographs of the left ankle joints of the experimental subjects in the various groups on the 21st day. It can be observed that swelling near the soft tissue, bone erosions and resorption in the periarticular bones was detected in the CFA-induced group. The narrowing of joint space was also observed in the subjects and a more significant effect was observed in members of the CFA group. However, the group treated with HEPE demonstrated dose-dependent protection from CFA-induced arthritic damage.

Synovial expression of pro-inflammatory cytokines by immunohistochemistry

Macrophage-derived inflammatory mediators, receptors and angiogenesis markers such as IL-1, IL-6 and NF-κB receptor are directly involved in the pathogenesis of RA and responsible for the hyperplasia of synovium and formation of pannus. Immunohistochemical staining of pro-inflammatory cytokines/cytokines receptors IL-1, IL-6 and NF-κB was carried out on the synovial tissue of CFA rats. As shown in Figure 9, immunohistochemical analysis of CFA-control group members demonstrated the massive protein expression of corresponding cytokines and transcription regulators IL-1, IL-6 and NF-κB. In contrast, IL-1, IL-6 and NF-κB protein expression was found to be lower in the group administered with HEPE in contrast to the control. Synovial expression of these markers in the indomethacin-treated groups was higher

than in the control groups but lower than in the HEPE-treated groups (200 mg/kg) (Figure 9).

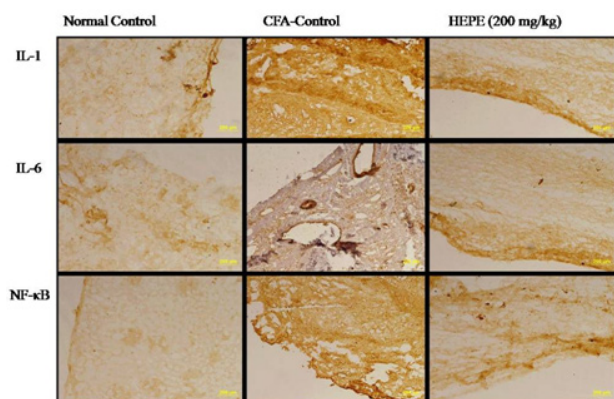


Figure 9. Effect of 200 mg/kg doses of CFA-control, Indomethacin and HEPE on immunohistochemical investigation of IL-1, IL-6 and NF- κ B in CFA-induced arthritis.

Discussion

The present study investigated whether oral administration of HEPE induces anti-arthritic or anti-inflammatory activity in carrageenan and CFA-adjuvant subjects. Furthermore, the effects of HEPE on macrophage inhibitory activity were assessed in a cotton pellet granuloma subject. To assess the immunological influences, the expression of pro-inflammatory (IL-1, IL-6) and NF- κ B were assessed. The prominent changes in histology of joints of all experimental animals were also explored.

As previously mentioned, RA is a provocative inflammatory disease the symptoms of which include swollen joints, an inflamed synovial membrane, bone erosion and destruction of cartilage.²⁶ Numerous mediators are involved in the onset and progression of diseases such as T lymphocytes, cytokines, neutrophils, B-cells, monocytes. IL-1 and TNF- α trigger self-proliferation of synoviocytes. Moreover, they increase production of tissue enzymes (matrix-metalloproteinases) resulting in the destruction of cartilage in joints.²⁰ It is stated in several reports that the imperative prevailing factors that induce inflammation, cartilage destruction and bone erosion are IL-1 and IL-6.²¹ The present study was, therefore, designed to examine the role of IL-1, IL-6 and NF- κ B in the attenuation of HEPE-induced bone damage in a CFA-induced model. A formaldehyde model was used to assess the preliminary anti-arthritic effect of HEPE extract and further confirmatory studies were carried out on a CFA-induced model. A cotton pellet-induced granuloma model assessed the macrophage inhibitory potential of HEPE extract, whereas a carrageenan model evaluated anti-inflammatory activity, thereby providing ground evidence to be tested for anti-arthritic activity. Acute and sub-acute toxicity studies conducted in keeping with OECD guidelines indicated the safe potential of the extract, while the LD₅₀ of HEPE was found to be greater than 500 mg/kg.

The decline in proinflammatory cytokine production

might contribute to one of the anti-inflammatory and anti-arthritic mechanisms of HEPE. Immunohistochemical observations confirmed the downregulation of NF- κ B by HEPE (200 mg/kg). Radiographical observations conducted during this study also proved that HEPE treatment reduced inflammation in CFA-induced models. Despite the protective role of HEPE with regard to inflammation in carrageenan-induced models, the severity of arthritis in CFA-induced subjects was also assessed by measuring changes in the joint diameter. It was established that a 200 mg/kg dose of HEPE significantly reduced the increase in joint diameter ($p < 0.001$) in CFA-induced rats. The effect of HEPE on joint histology and immunohistochemical analysis of IL-1, IL-6, and NF- κ B was studied to investigate the effect on pro-inflammatory cytokines/receptors. The dysfunction in ankle joints was assessed by means of radiographic examination and the damage to the subchondral bone was investigated using a scoring system as described by Karwasra *et al.*²⁶ Histopathological and radiographic analysis confirmed that primarily tarsals, metatarsals, and phalanges of HEPE at higher doses exhibited protection against CFA-related changes. The radiographical score markedly increased in the CFA-control group, whereas HEPE significantly lowered the radiographical score in the synovium of CFA-induced subjects. These figures established that HEPE alleviates cartilage destruction, swelling, inflammation, and oxidative stress in Complete Freund's adjuvant-induced subjects. Finally, it was demonstrated that HEPE effectively ameliorated experimental arthritis by attenuating pro-inflammatory cytokines via downregulation of NF- κ B. Taken together, this initial attempt demonstrated that HEPE induces anti-arthritic activity which could be utilized in the treatment of RA.

Conclusion

HEPE constitutes a potential therapeutic target for RA, increasing its role in the pathogenesis of inflammation and bone damage. The study conducted was beneficial in providing validated information on HEPE as a complementary and alternative approach. It alleviates numerous symptoms of inflammation, inhibition of macrophage activity and modulate IL-1, IL-6 and NF- κ B in RA.

Ethical Issues

The investigative procedure was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) (approval number: 760/IAEC/13).

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Conflict of Interest

The authors declare they have no conflict of interest.

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