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# Evaluation of antiarthritic activity of isoeugenol in adjuvant induced arthritis in murine model

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ABSTRACT

Isoeugenol, a component of clover oil, possesses potent anti-inflammatory and antioxidant activity. In the present study, we investigated the effect on experimentally induced adjuvant arthritis in rats. Induction of arthritis in adjuvant exposed rats was confirmed by appearance of several physical symptoms such as redness, swelling and stiffness of paws, radiographic analysis revealing joint damage, soft tissue swelling of the footpad, histopathologic changes and expression of proinflammatory enzymes and mediators in the joint tissue. Treatment of rats with isoeugenol, however, conferred a significant protection against almost all the investigated parameters. Isoeugenol significantly and dose dependently attenuated arthritic index, paw circumference, joint stiffness and the levels of proinflammatory mediators. Exposure to isoeugenol inhibited the release of nitric oxide and proinflammatory cytokines the including $PGE_2$ and TNFα from lipopolysaccharide primed macrophages. Isoeugenol also showed a significant analgesic activity in acetic acid-induced writhing model. Further, unlike most antiarthritic drugs, isoeugenol had no damaging effect on gastric mucosa, which makes it a favorable antiarthritic drug. Thus, the results obtained in the present study indicate isoeugenol to possess a promising antiarthritic activity and further advocate the efficacy of natural products as antiarthritic therapeutics.



## 1. Introduction

Arthritis is a chronic, deforming disease of the joints of unexplained etiology. It is characterized by destructive changes in the cartilage and bone, and by bony outgrowths restricting mobility of the joint.

Inflammation is a key player in the pathophysiology of arthritis. The activated leukocytes and synovial fibroblasts in the joint tissue secrete several proinflammatory mediators such as TNF-α, IL-1, IL-6, IL-8, PGE2, INF-γ etc. to cause inflammation and joint degradation (Wright et al., 2010). Leukocytes near the synovium induce the expression of proinflammatory enzymes, which may further activate them by autocrine and paracrine signaling and stimulate the synovial fibroblasts to release matrix metalloproteinases such as MMP-1 and MMP-9 that may erode cartilage and bone. Thus, the potential strategies to impede arthritis may include blockage of release of proinflammatory mediators and matrix metalloproteinase from leukocytes and synovial fibroblasts.

Arthritis is currently one of the most prevalent diseases worldwide. However, unfortunately, till date there is no specific cure of arthritis. The current therapeutics for the disease are focused on alleviation of inflammation, pain and joint damage. These include nonsteroidal anti-inflammatory drugs (NSAIDs), specific inhibitors of proinflammatory mediators, glucocorticoids etc. However, most of these drugs possess a wide spectrum of untoward effects (Sooriakumaran, 2006; Simadibrata, 2004). Treatment of arthritis with herbal agents is probably safer. Due to natural origin, herbs are considered to have minimum or no side effects. Herbal agents also are pleiotropic, i.e.; they act on multiple loci to thwart arthritis. Curcumin, for instance, has been shown to hinder many aspects of arthritis. It decreases the activation of T and B cells, macrophages, neutrophils and natural killer cells (Jagetia and Aggarwal, 2007); inhibits NFκB downregulating the expression of inflammatory cytokines (Jagetia and Aggarwal, 2007), induces apoptosis in synovial fibroblasts (Park et al., 2007) etc. It also possesses gastroprotective activity (Rivera-Espinoza and Muriel, 2009). Pleiotropy makes herbal agents the drugs of choice since arthritis is a multifactorial process and demands multifactorial treatment.

Thus, apparently there are several reasons to explore plant based antiarthritic medicines. In the present study, we investigated the antiarthritic potential of isoeugenol (4-propenyl-2-methoxyphenol), which is a naturally occurring phenolic constituent of clove oil, monkey orange, basil etc. It is commonly used as an antioxidant and flavoring agent in food products. It possesses antiinflammatory activity (Li et al., 2006). Its dimmer, dehydrodiisoeugenol, inhibits lipopolysaccharide stimulated nuclear factor kappa B activation

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and cyclooxygenase-2 expression in macrophages (Murakami et al., 2005). Isoeugenol may therefore be expected to effectively inhibit arthritis. Thus, in the present study, we investigated the efficacy of isoeugenol against arthritis exploring its effect on cartilage and bone damage, and release of chief proinflammatory mediators implicated in the pathophysiology of arthritis.

## 2. Materials and methods

### 2.1. Chemicals

Thiobarbituric acid (TBA), RPMI-1640 media, isoeugenol, reduced nicotinamide adenine dinucleotide (NADH) was purchased from Sigma chemical company, USA. Complete Freund Adjuvant (CFA) was purchased from Difco laboratories, MI, USA. TNF-α and PGE2 ELISA kits were purchased from ebioscience and Cayman chemical company respectively. Antibodies for COX-2 and iNOS were procured from Biovision. All the other chemicals used were purchased from local companies and were of highest available purity grade.

### 2.2. Induction of arthritis

Male Wistar rats (six to seven weeks old weighing 130–150 g) procured from Central Animal House, Jamia Hamdard were used throughout this study. Animals were housed in an air-conditioned room and had a free access to water and pellet diet. The animal handling and experimental procedures were carried out according to the guidelines of the institutional animal ethics committee (IAEC) accredited by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India. Rats were divided into five groups: group 1 to group 5, of six rats each. Group 1 comprised of saline treated control. Rats of groups 2–5 were administered 0.1 ml of the adjuvant intradermally. Maximum amount of paw edema was observed on day 14 following adjuvant administration. From day 1 to 14, rats of group 3 were daily administered 10 mg/kg body wt. p.o. isoeugenol, rats of group 4 were administered 25 mg/kg body wt. p.o. isoeugenol and rats of group 5 were administered 3 mg/kg body wt. p.o. indomethacin (used as positive control). Thereafter, these animals were evaluated for arthritis on day 24. The percentage of inhibition of arthritis was expressed as the reduction in paw volume with respect to the control group. Following radiographic and physical evaluation, rats were sacrificed by cervical dislocation and blood and paws were isolated for further analysis.

Arthritic index (AI) scores were assigned to ankle joints of the rats of each group. Scoring was performed on a 0–4 scale as follows: 0 = no swelling or erythema, 1 = slight swelling and/or erythema, 2 = low to moderate edema, 3 = pronounced edema with limited joint usage, and 4 = excess edema with joint rigidity. Circumference of paw was determined, for which two perpendicular diameters of the paw were measured with a caliper. The circumference was determined using the geometric formula: circumference = $2\pi$ ($\sqrt{}$ ($a^2 + b^2/2$)), where $a$ and $b$ are the two diameters. The scoring of joint stiffness was also performed, for which the body of rats was held from the back and the bending and extension (once in each direction) of the ankle within its limits of range of motion was evaluated. It was confirmed beforehand that there was no restriction of ankle joint movement in the bending and extension manipulations in naive rats, and the scoring was performed according to the evaluation scale reported by Butler et al. (1992).

*Score 2:* when there was restriction of full range of movement of the ankle in both bending and extension.

*Score 1:* there was restriction of full range of movement of the ankle in either bending or extension.

*Score 0:* no restriction.

### 2.3. Radiographic analysis

The rats were anesthetized and placed on a radiographic machine at a distance of 90 cm from the X-ray source. Radiographic analysis of hind paws was performed with a 40 kW exposition for 0.01 s. Rats were considered for the following radiograph parameters: soft tissue swelling, articular cartilage damage, and osteophyte formation, with the following criteria and scores: 0 = no damage, 1 = mild, 2 = moderate and 3 = severe.

### 2.4. Histology and immunohistochemistry

Hind paws were fixed in 10% phosphate buffered formalin for 24 h, dehydrated and then embedded in paraffin. Four-μm thick sections were cut and visualized by routine H & E staining.

For immunohistochemistry, formalin-fixed, paraffin-embedded sections were deparaffinized, subjected to antigen retrieval by heat treatment in citrate buffer and then subjected to peroxidase block by exposing to 3% $H_2O_2$. Target molecules (iNOS and COX-2) were detected in the sections by incubating the slides with appropriately diluted primary antibody specific to target molecule at room temperature for 1 h in Tris-buffered saline containing and 0.05% Tween-20 and then developing with the HPR conjugated secondary antibodies. The peroxidase binding sites were detected by staining with 3,30-diaminobenzidine tetrahydrochloride. The slides were finally counter-stained with hematoxylin.

### 2.5. Evaluation of TNF-α, $PGE_2$ and nitrite in Paw

Fifty percentage homogenate of paw tissue was prepared in 2.5 ml of 10 mM HEPES buffer (pH 7.4) containing 0.32 M sucrose, 100 μM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 100 μM leupeptin. Homogenate was centrifuged at 2000*g* for 15 min at 4 °C. TNF-α and $PGE_2$ levels were measured in the supernatant by ELISA kit according to instructions of the manufacturer. To measure nitrite levels, 0.2 ml of supernatant was mixed with equal volume of griess reagent [0.1% napthylethylenediamine plus 1% sulphanilamide (prepared in 5% phosphoric acid)]. The absorbance was measured at 510 nm.

### 2.6. Nitric oxide, prostaglandin $E_2$ ($PGE_2$) and tumor necrosis factor alpha (TNF-α) in cultured rat peritoneal macrophages

Rat peritoneal macrophages were isolated and cultured according methods previously described (Kaur et al., 2004). Macrophages were stimulated with 10 μg/ml lipopolysaccharide (LPS) for 24 h at 37 °C in the presence or absence of different concentrations of isoeugenol. The amount of nitrite released was determined by mixing 0.5 ml of culture supernatant with 0.5 ml of Griess reagent and reading at 510 nm. The levels of $PGE_2$ and TNF-α in the supernatant of the culture medium were measured by ELISA assay kits by following manufacturer's instructions.

### 2.7. Analgesia

Swiss albino mice (weighing 25–30 g) were used for this study. Mice were administered 1% acetic acid solution (0.1 ml) intraperitoneally. 50 and 150 mg/kg body weight isoeugenol were administered orally 1 h before administration of acetic acid. Control mice were administered the vehicle in the same experimental conditions. Following acetic acid injection, each mouse was observed for next 20 min for recording the numbers of instances of writhing and stretching.

### 2.8. Gastric mucosal lesions

Mice (Swiss albino, weighing 25–30 g) were fasted for 18 h before the experiments, but had free access to water. Isoeugenol or indomethacin (used as standard) was orally administered to mice. Mice were sacrificed after 4 h. Their stomachs were removed, inflated by injecting 7 ml of 2% buffered formalin, placed in 4% buffered formalin for 10 min to fix the gastric wall and then cut opened. The mucosal surface was washed with normal saline and the lesions developed in mucosa were analyzed under a dissecting microscope. Damage to the mucosa was scored from 0 to 4 on an arbitrary scale as follows: 0 = no lesions; 0.5 = hyperemia; 1 = one or two lesions; 2 = severe lesions; 3 = very severe lesions; 4 = mucosa full of lesions.

## 3. Results

### 3.1. Effect of isoeugenol on adjuvant induced arthritis

Arthritis was evident in rats after 2 weeks of adjuvant administration. Significant increase in paw circumference, erythema, swelling, joint stiffness and hindrance in the movement was observed. The arthritic index of this group was 3.8 (Table 1). All the rats of group 2 developed arthritis after 24 days. However, in rats that received isoeugenol, a significant decrease was observed in paw circumference (Fig 1), erythema, swelling and joint stiffness (Table 1). The arthritis index of this group was 1.8 (Table 1).

Radiography of rats in which arthritis was induced by adjuvant exposure revealed intense periarticular inflammation, soft tissue swelling, bone resorption and joint erosion (Fig 2b). However, in

**Table 1**
Effect of Isoeugenol on articular index of CFA induced arthritis in rats. Results are expressed as mean ± S.E. ($n$ = 6).

| Group | Arthritic index | Stiffness score |
|---|---|---|
| Control | 0 | 0 |
| CFA induced arthritis | 3.8 | 2.0 |
| CFA + Isoeugenol (10 mg/kg b wt.) | 2.8 | 1.5 |
| CFA + Isoeugenol (25 mg/kg b wt.) | 1.8 | 0.75 |
| CFA + Indomethacin (3 mg/kg b wt.) | 1.8 | 0.75 |