EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH
www.ejpmr.com

EMBELIN FROM *EMBELIA RIBES* SUPPRESSES CARTILAGE DAMAGE AND AMELIORATES INFLAMMATION IN AN EXPERIMENTAL RAT ADJUVANT INDUCED ARTHRITIS MODEL

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ABSTRACT

The Embelin a quinone derivative (*3-undecyl 2,5-dihydroxy, 1,4-benzoquinone*) from the fruit of the Embelia *ribes* Burm. plant (Myrsinaceae) called false black pepper has been proven to exhibit antihelmintic, antifertility, antitumor, antimicrobial, analgesic and anti-inflammatory activity. The present study was undertaken to investigate the therapeutic potential of Embelin, on adjuvant arthritis in rat as well as the possible mechanisms. Adjuvant arthritis (AA) was induced in rats on day 0. Embelin 10, 30 and 50 mg kg^{-1} day^{-1}, or prednisone 10mgkg^{-1} day^{-1} was given to rats orally from day 19 to day 24. Embelin significantly inhibited paw swelling and bone destruction in AA rats. Serum level of IgG anti-Mycobacterium tuberculosis antibodies and delayed-type hypersensitivity (DTH) induced by *Mycobacterium tuberculosis* were also decreased by Embelin. The effects of Embelin were associated with decreased interleukin-1β (IL-1 β) mRNA expression in ankle joint synovial membrane and tumor necrosis factor-α (TNF--α) mRNA expression in homogenized paws along with histological and radiological analysis from adjuvant-induced arthritic rats. These findings suggested that Embelin had a therapeutic effect on adjuvant arthritis.

KEYWORDS: Mycobacterium tuberculosis antibodies and delayed-type hypersensitivity.

1. INTRODUCTION

Rheumatoid arthritis (RA) is a systemic and chronic inflammatory autoimmune disease characterized by chronic inflammation, synovial hyperplasia with concomitant joint destruction, deformity and loss of function.[1] Although the exact pathogenesis and etiology of the disease remain unclear, the main pathological changes have been defined, such as abnormal immunity, chronic synovitis, inflammatory cell infiltration, pannus formation, destruction of cartilage and bone erosion. The development of RA involves a complex interplay of several types of cells, including B and T lymphocytes, macrophages, fibroblast-like synoviocytes, endothelial cells and dendritic cells. Notably, B and T cells play critical roles in the pathogenesis. Currently, the clinical need for effective treatment of RA remains unmet and more novel drugs are highly demanded.

Rat adjuvant arthritis (AA) is a chronic, polyarticular, erosive type of arthritis induced by an injection of killed mycobacteria[2] AA in rat is an experimental model that shares some features with human rheumatoid arthritis (RA). One of the most important features of AA is the chronic synovitis, including inflammatory cell infiltration, pannus formation, cartilage destruction and bone erosion. AA is widely used for studying the pathogenesis of RA and for searching new drugs for treatment of rheumatoid disease.[3,4,5,6]

The fruit of the *Embelia ribes* Burm. plant (Myrsinaceae) (called false black pepper in English, Vidanda in Sanskrit, and Babrang in Hindi languages) contains a quinone derivative Embelin (3-undecyl 2,5-dihydroxy, 1,4-benzoquinone) as its active constituent. Embelin is reported to have anthelmintic, antifertility[7] antitumour, antimicrobial, analgesic anti-inflammatory and anti-diabetic activity. Although the precise mechanisms underlying most of these activities are unclear, recent evidence indicate that the antitumor activity can attributed to the ability of embelin to bind to and inhibit XIAP,[8] thereby inducing activation of capsace3, -7 and -9, and apoptosis. Embelin is also reported to block nuclear kappa factor- κB signaling pathways, thereby leading to down-regulation of a variety of gene products involved in tumor cell cervical, proliferation, invasion, angiogenesis and inflammation.[9] TNF- α can activate NF-κB by degrading IκB, its inhibitory protein. However, the effect of embelin on (Rheumatoid arthritis) is not known.[10,11] Hence the aim of the present study was to investigate the effect of embelin on Rat adjuvant arthritis (AA) inflammation in rats.

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Authentication

Embelia ribes fruit were collected from B. V. Patel Pharmaceutical Education & Research Development (PERD) Centre, Gujarat, India. Their authenticity was confirmed by the taxonomist of Phytochemistry Department and voucher specimen was deposited at the Department of Plant biology and Plant Biotechnology Presidency College, Chennai, India. Twenty five grams of *Embelia ribes* fruit was ground with 100 ml x 4 petroleum ether under reflux for 30 min at a maximum temperature of 50ºC. The extract was filtered, concentrated to one-third volume and kept overnight at room temperature. After decanting the supernatant, an orange colored mass was obtained from the bottom, this is the Embelin fraction. It was purified by repeated crystallization with methanol having a melting point of 142-143°C, to obtain glistening orange crystals of pure Embelin. The structure was confirmed by IR, 1^H NMR and mass spectral.

2.2 Animals

Male Wistar rats weighing 160±20 g were obtained from animal house of C.L. Baid Metha College of Pharmacy, Chennai and were acclimatized to the housing conditions for 7 days with access to laboratory chow diet and water *ad libitum*. Animal requirement was approved by the Institute of Animal Ethics Committee, and all the experiments were conducted as per the norms of the committee for the purpose of supervision of experiments on animals.

2.3 Drugs and reagents

Prednisone was obtained from Sigma-Aldrich India. Embelin or prednisone was ground and suspended in normal saline containing 0.5% sodium carboxymethyl cellulose (CMC) for administration. Embelin or prednisone was ground and suspended in normal saline containing 0.5% sodium carboxymethyl cellulose (CMC) for administration.

2.4 Induction of adjuvant arthritis

Adjuvant arthritis was induced by an injection of Complete Freund's adjuvant, M. tuberculosis H-37 RA, 5 mg/ml, desiccated in 100^{-1} mineral oil from Chondrex Inc, into the base of tail (day 0). Sixteen days after inoculation, the animals were selected and distributed into groups (n = 10) according to the severity of arthritis, so that each group had similar disease severity at the beginning of the treatment. One was given 0.5% CMC solution as vehicle-treated group, the others were given embelin (10, 30 and 50mgkg $^{-1}$ day^{-1}), or prednisone (10mg kg^{-1} day^{-1}) intragastrically from day 19 to day 24, respectively.

2.5 Quantification of paw edema

The severity of AA was quantified by measuring the volume of hind paws using a water plethysmometer. Paw volume (ml) was measured on days 0, 16, 18, 20, 22, 24, 26 and 28 after arthritis induction. Data were expressed as the volume of increase with respect to day 0 volume.

2.6 Blood sampling and anti-Mycobacterium IgG antibody quantification

Adjuvant arthritis was elicited in rats and treatment was given as described above. Rats were sacrificed on day 26 after arthritis induction and blood samples were obtained. Serum was stored at −70 ◦C until use. IgG anti-*Mycobacterium tuberculosis* antibodies were determined by enzyme-linked immunosorbent assay (ELISA)[6] Briefly, 96-well plates (Costar, Corning, NY) were coated with 0.1ml soluble fraction of *Mycobacterium tuberculosis* of 3µg of protein/ml prepared in 0.15M phosphate-buffered saline (PBS) and incubated for 2 h at room temperature in a moist chamber. Free sites of the plastic surface were blocked with PBS containing 0.05% Tween 20 and 1% bovine serum albumin (BSA). Serum samples were diluted at 1:200 in PBS containing 0.05% Tween 20 and 1% BSA, and added to plates. Then, plates were washed with PBS containing 0.5% Tween 20 and 100µl goat anti-rat IgG (whole molecule) -peroxidase (Zhongshan Golden Bridge Biotechnology Co., Ltd.), prepared in PBS containing 0.05%Tween 20 and 1% BSA diluted at 1:2500, was added. The plates were washed with PBS containing 0.5% Tween 20. After the assay was developed with *o*-phenylenediamine, the plate was incubated for 30min at room temperature. Sulfuric acid (50µl, 2M) was added to every well and the optical density was measured at 492 nm using a well scanner ELISA reader (Softmax).

A pooled standard batch of sera from arthritic animals was used as positive control on every plate and used to calculate relative unit, which was expressed as U/ml, for the anti-Mycobacterium antibody response.

2.7 Delayed-type hypersensitivity (DTH)

DTH was induced on day 23 after AA induction by an intradermal injection into the left ear of 20µl of a 100 µg/ml soluble fraction of *Mycobacterium tuberculosis*.[6] The right ear was injected with normal saline as a control. After 24 h, the thickness of the pinna was measured with a dial thickness gauge (Mitutoyo,Japan). The non-specific increase in ear thickness was also determined by injecting 20µl soluble fraction of *Mycobacterium tuberculosis* in the left

ear of normal rats. Results are expressed as a percentage of increase in the thickness of the left ear.

2.8 Hind limb mRNA extraction and cytokine mRNA polymerase chain reaction analysis (PCR)

Adjuvant arthritis was elicited in rats and treatment was given as described above. On day 26 of arthritis, four animals from each group were killed and the hind left limbs were amputated at about the ankle. Frozen synovial membranes of ankles and hind paws were crushed and homogenized in liquid nitrogen and total RNA was extracted using Beyozol reagent (Beyotime Biotechnology) according to the manufacturer's recommendation. The RNA was reverse transcribed into cDNA with random hexamers (Promega Corporation). Then PCR was performed in a total volume of 50µl containing 3 units of rTag and 50 pmol of primers specific for murine interleukin-1β (5$^{/}$-CAG CTA CCT ATG TCT TGC CC-3$^{/}$, 5$^{/}$-GTC GTT GCT TGT CTC TCC TT-3$^{/}$), for GAPDH (5$^{/}$-GTG GGG CGC CCC AGG CAC CA-3$^{/}$, 5$^{/}$-CTC CTT AAT GTC ACG CAC GAT TTC-3$^{/}$) and for murine tumor necrosis factor-α (5$^{/}$-ACC CCC AAC CTA TGA AGA AA-3$^{/}$, 5$^{/}$-TCC ACG CAA AAC GGA ATG AA-3$^{/}$). Cycling conditions were as follows: 30 s of denaturation at 94°C, 1 min of annealing at 61°C and 1.5min of elongation at 72°C for 40 cycles. Equal volume products of reverse transcription-polymerase chain reaction (RT-PCR) were separated on an agarose gel (1.5%) and visualized with ethidium bromide staining by a gel documentation system. The extent of interleukin-1β or tumor necrosis factor-α expression was quantitated using a densitometer with multi gauge software (Gel Doc GIS-2008,Biotech ,Chennai). Glyceraldehyde phosphate dehydrogenase (GAPDH) levels were also analysed as a control. Sample PCR product values of interleukin-1β or tumor necrosis factor-α were expressed as a percentage of the density of GAPDH in the same sample.

2.9 Histological examination

Rats were sacrificed on day 29 after immunization. The legs and hind paws were removed, fixed with 10% formaldehyde in normal saline, and then decalcified for 10 days with ethylene diamine tetra acetic acid and embedded in paraffin for histological analysis. The paraffin sections were stained with hematoxylin and eosin. Ankle joints were examined. The histopathological alteration of joints was blindly graded by a pathologist and assigned a score of 1–4 based on the following criteria: 1, minimal synovitis, primarily infiltration of mononuclear inflammatory cell into synovial membrane; 2, mild synovitis, pannus formed,

cartilage degeneration; 3, proliferation and infiltration of a large amount of mononuclear cells, subchondral bone erosion; superficial cartilage damage; 4, severe destruction of cartilage and subchrondral, complete disorganization of the joint space, fiber thickening and severe fibrosis, bony ankylosis.

2.10 Radiological analysis

Before sacrificing the animals, X-rays were taken at the joints of the hind paw of the animals for evaluating the bone damage. Radiographs were taken using X-ray apparatus Kodak Diagnostic Film, Ready-Pack, X-OMATk, Kodak, NY, USA) using a MBR-1505R (Hitach Medical Corporation, Tokyo, Japan). The settings for radiographs were 5 mA, 50 kV and 1 min exposure. Films were placed 60 cm below the X-ray source.

2.11 Statistical analysis

Quantitative variables were expressed as mean±S.D. One-way analysis of variance (ANOVA) was used. If any significant change was found, *post hoc* comparisons were performed using Graph prism 5. Non-normal and ranked distribution data were analysed by method of Mann–Whitney *U*-test. *P*-Values < 0.05 were considered significant.

3.   RESULTS

3.1. Effects of Embelin on hind paw swelling in adjuvant arthritis rats

Arthritis was induced reproducibly in all animals injected the adjuvant, with onset of injected hind paw (right paw) erythema and swelling (arthritis onset) occurring on day 9, swelling of non injected hind paw (left paw) began on day 11 and persisted to the end of the experiment. Treatment with embelin (10, 30, 50 mg kg^{-1} day^{-1}, days 19–24) and prednisone (10 mg kg^{-1} day^{-1}) diminished the right hind paw swelling from day 20 to 28 ($P < 0.05$–0.001) and left hind paw swelling from day 24 to 28 after immunization ($P < 0.05$–0.001) (Fig. 2A and B). Maximum inhibitory rates in the drug-treated groups were 28.4%, 54.1%, 71.4% (Embelin 10, 30 and 50 mg kg^{-1} day^{-1}) and 68.3% (prednisone 10 mg kg^{-1} day^{-1}) for the right paw (injected), and 46.3%, 71.2%, 80.2% (Embelin 10, 30 and 50 mg kg^{-1} day^{-1}) and 80.1% (Prednisone 10 mg kg^{-1} day^{-1}) for the left paw. The reduction of edema was sustained throughout the experiment even the treatment ended on day 24. These *in vivo* results demonstrated that embelin was effective in suppressing the development of AA in rats.

3.2. Effects of embelin on delayed-type hypersensitivity and anti-Mycobacterium IgG antibody

Ear thickness was significantly increased in the vehicle treated adjuvant arthritis group compared with the normal group ($P < 0.001$). Embelin significantly decreased the delayed-type hypersensitivity induced by Mycobacterium at all three doses assayed ($P < 0.05$–0.001) (Fig. 3A). In the vehicle-treated adjuvant arthritis group, serum levels of IgG anti-Mycobacterium antibodies were significantly increased compared with the normal group ($P < 0.001$) (Fig. 3B). Embelin 20 mg kg^{-1} day^{-1} significantly decreased anti-Mycobacterium antibodies as compared with the vehicle-treated adjuvant arthritis group ($P < 0.05$) (see Fig. 3B).

3.3. Effects of Embelin on mRNA expression

While interleukin-1β. mRNA was detectable in synovial membrane, tumor necrosis factor-α. mRNA could not be detected at all. The antisense primer is all right because it successfully gave tumor necrosis factor- α. mRNA in hind paw. In the vehicle-treated adjuvant-induced group, interleukin-1β . mRNA in synovial membrane and tumor necrosis factor-.α mRNA in hind paw was significantly increased compared with the normal group ($P < 0.001$). Embelin (10, 20 and 50 mg kg^{-1} day^{-1}) and prednisone significantly decreased the interleukin-1β. mRNA expression when compared with the vehicle-treated adjuvant induced group ($P < 0.05$–0.001) (Fig. 4A). All three doses of Embelin and prednisone inhibited the tumor necrosis factor-α mRNA expression in hind paw ($P < 0.01$–0.001) (Fig. 4B).

3.4. Effect of Embelin on joint destruction in adjuvant arthritis

The histological architecture of the joint was markedly abnormal in the model group rats (Fig. 5B), which showed pronounced synovial hyperplasia and inflammation, increased numbers of vessels (angiogenesis), and extensive erosive changes in the cartilage and bone. In contrast, embelin 50 mg kg^{-1} day^{-1} or high dose of embelin treated rats preserved a nearly normal histological architecture of the joint, with mild focal to little synovial hyperplasia, significantly reduced numbers of synovial vessels and inflammatory cells, and diminished erosive changes in the cartilage and bone. Meanwhile, the histological scores of tissue sections from rat ankles in embelin 10, 30 and 50 mg kg^{-1} day^{-1} treated groups were reduced dose-dependently. These histological findings supported the clinical observations and further demonstrated the therapeutic effects of embelin on rat AIA (Table 1).

3.5. **Radiological Evaluation of Adjuvant Induced Arthritis**

Radiographic severity of joint destruction is shown in (Fig.6B) right and left ankle joints of arthritis control rats, severe bone destruction and deformation and joint space narrowing was detected**.** The destruction of right ankle joints was more severe than that of left joints. In both ankle joints, embelin (50 mg kg^{-1} day^{-1}) markedly inhibited bone destruction as shown in (Fig.6E). Scoring of radiological destruction in ankle joints was performed on the basis of bone destruction or joint space narrowing. Bone destruction of both ankle joints in embelin 50mg/kg treated rats was significantly reduced when compared to arthritis control rats. Prednisone 10 mg kg^{-1} day^{-1} tended to reduce the scores of joint space narrowing in ankle joints. Both in bone destruction and in joint space narrowing, effects of embelin 50 mg kg^{-1} day^{-1} on joints were more marked than other two doses.
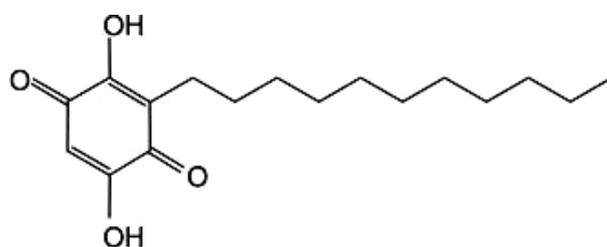
Embelin [MW 294]
(2,5-Dihydroxy-3-undecyl-1,4-benzoquinone)
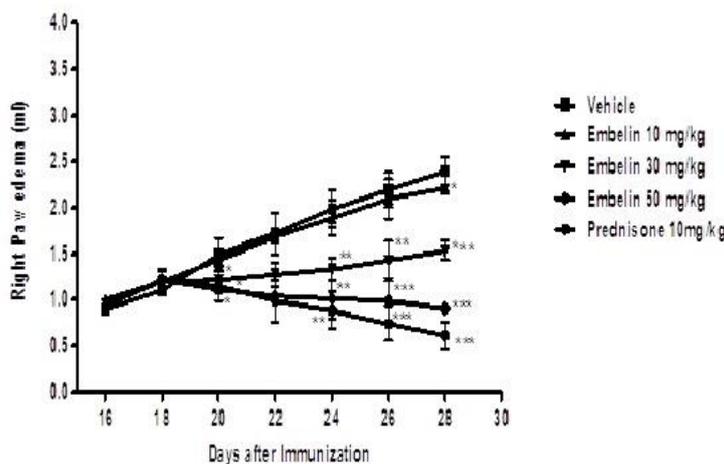
Fig.1. Structure of Embelin
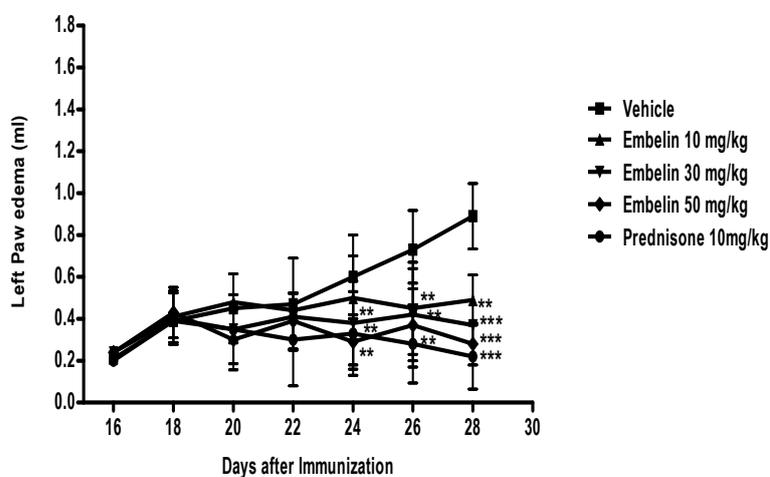
Fig. 2. Effect of embelin on the development of adjuvant arthritis in rats. Rats were immunized with Freund's complete adjuvant on right paw on day 0. Immunized rats were grouped randomly and treated with embelin 10, 30 and 50 mg kg^{-1} day^{-1}, prednisone 10 mg kg^{-1} day^{-1} or vehicle from day 19 to day 24; rats were sacrificed on day 29. Data expressed as mean ± S.D.; n = 10 rats for each group. *P < 0.05, **P < 0.01,***P < 0.001 vs. vehicle-treated group, tested by Mann–Whitney U-test. Right paw edema progression (A), left paw edema progression (B)

(A)

(B)
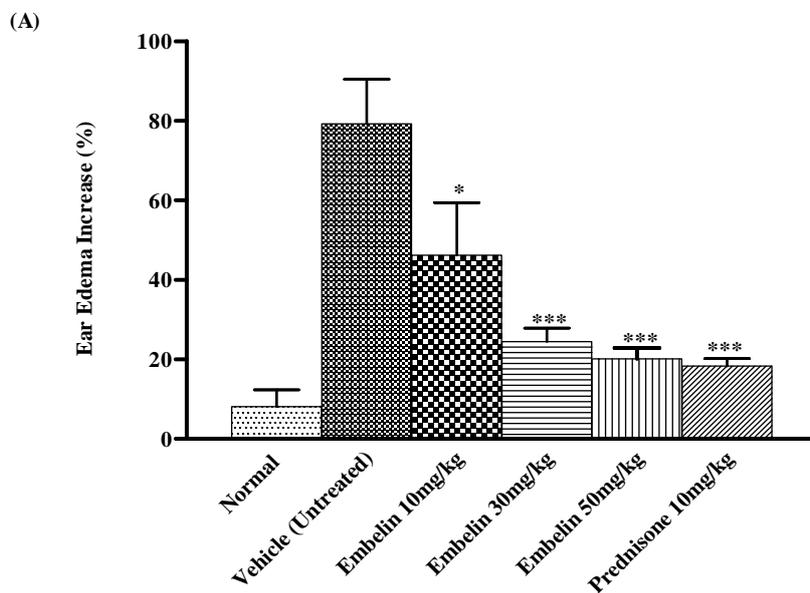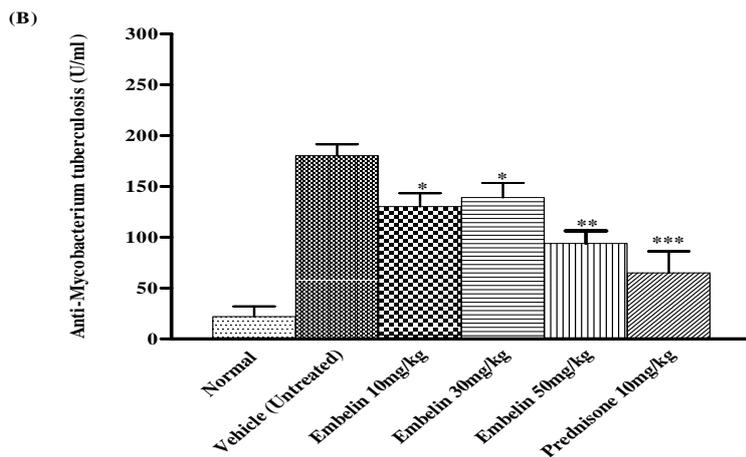
Fig. 3. Effect of Embelin on delayed-type hypersensitivity and on serum levels of IgG anti-Mycobacterium tuberculosis antibodies. Rats were immunized with Freund's complete adjuvant on right paw on day 0. Immunized rats were grouped randomly and treated with Embelin 10, 30 and 50 mg kg^{-1} day^{-1}, prednisone 10 mg kg^{-1} day^{-1} or vehicle from day 19 to day 24. On day 22, rats were hypodermic injected 100 .g/ml Mycobacterium tuberculosis 20 µl on left ear and normal saline 20µl on right ear. Twenty-four hours later, the thickness of the pinna was measured. The results were expressed as a percentage of increase in the thickness of the left ear with respect to the right ear (A). Blood samples were obtained on day 26 after arthritis induction and levels of antibodies in serum were determined by ELISA (B). Data expressed as mean ± S.D.; n = 10 rats for each group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. vehicle-treated group, tested by Mann–Whitney U-test.
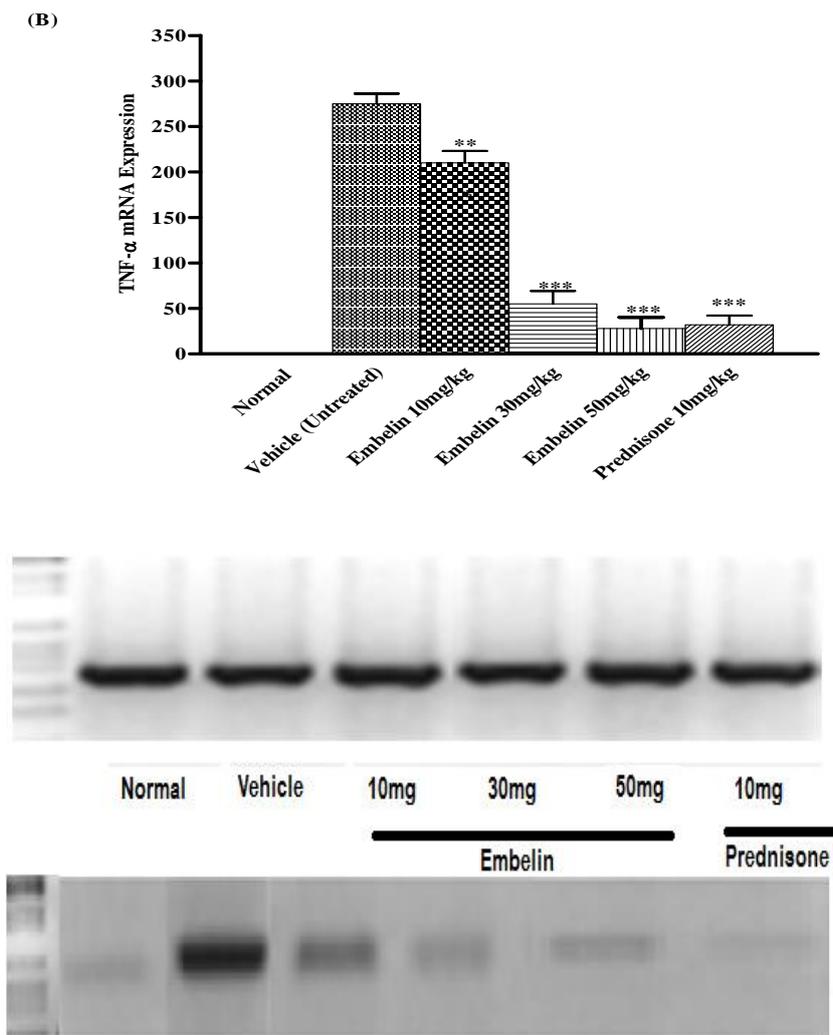
(A)
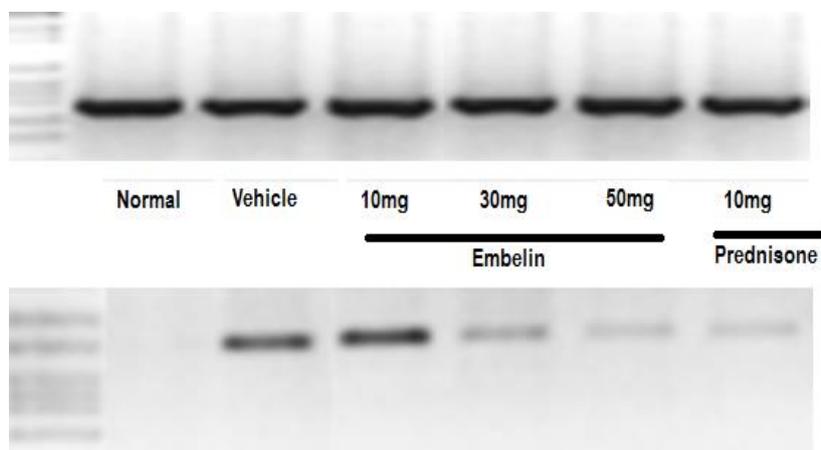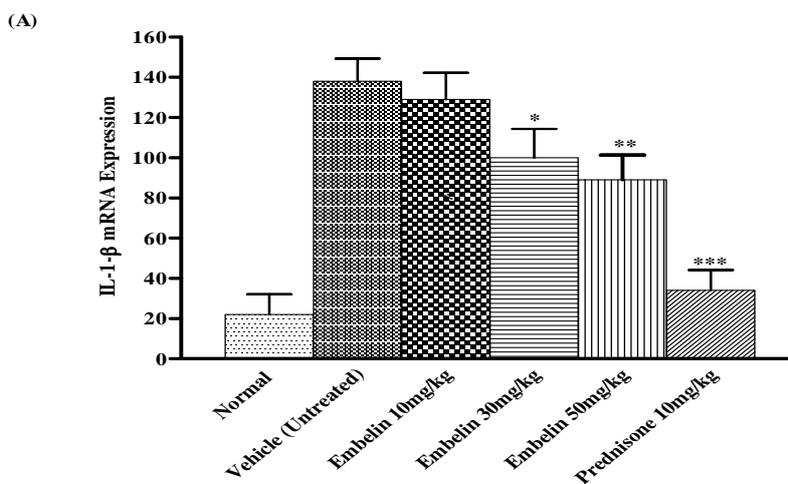
Fig. 4. Effect of Embelin and prednisone on interleukin-1β mRNA in synovium (A) and TNF-.α mRNA in hind paw (B). Rats were immunized with Freund's complete adjuvant (FCA) on right paw on day 0. Immunized rats were grouped randomly and treated with Embelin 5, 10 and 20 mg kg^{-1} day^{-1}, prednisone 10 mg kg^{-1} day^{-1} or vehicle from day 19 to day 24. mRNA expression of IL-1β and TNF-α were expressed as a percentage of β-actin (n = 4 rats for each group). Data expressed as mean ± S.D.;*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. vehicle-treated group, tested by ANOVA and Fisher's PLSD.
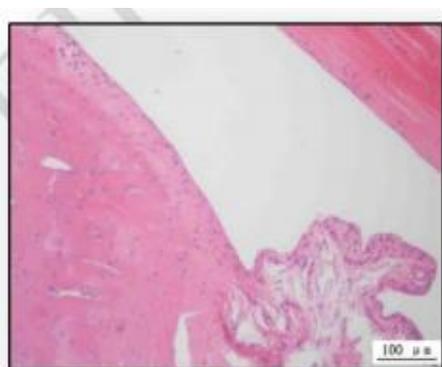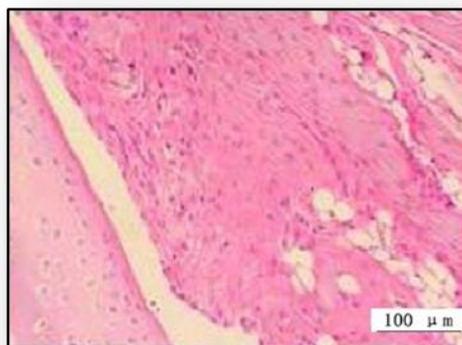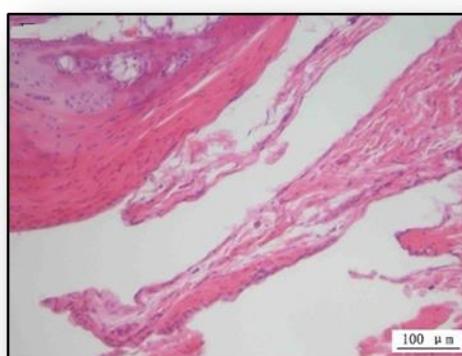

(A)Normal   **(B) Vehicle**
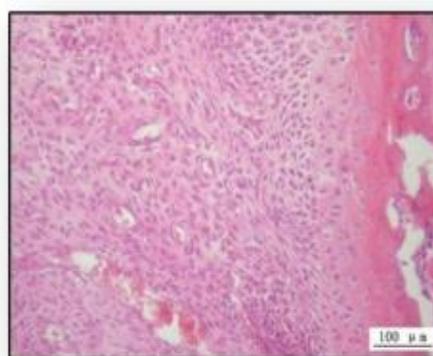
(C) Embelin (10mg/kg)   **(D) Embelin (30mg/kg)**
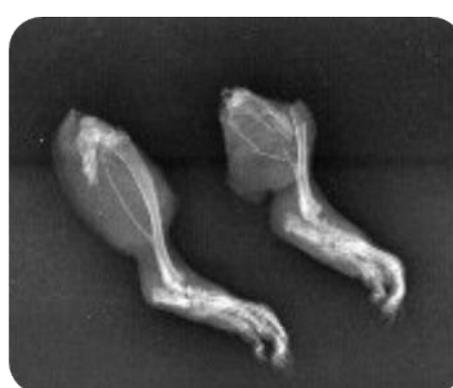

(E) Embelin (50mg/kg)   **(F) Prednisone (10mg/kg)**

Fig. 5. The extent of foot pathological lesions was graded on a semiquantitative scale. Light microscopy 100×. Joint tissue was fixed in 3% formaldehyde and 5µm paraffin sections were stained with hematoxylin and eosin (A–F). (A) Normal (B) Vehicle (Untreated) (C) Embelin(10mg/kg), (D) Embelin(30mg/kg), (E) Embelin(50mg/kg), (F) Prednisone (10mg/kg).
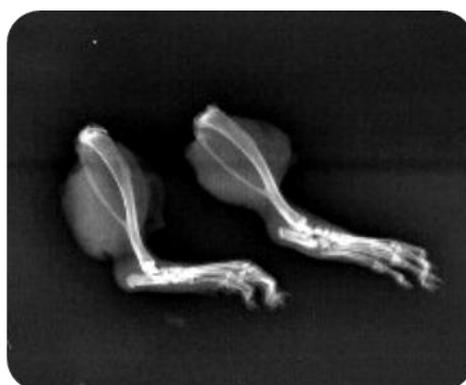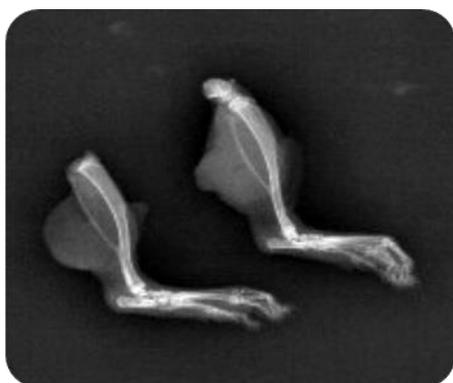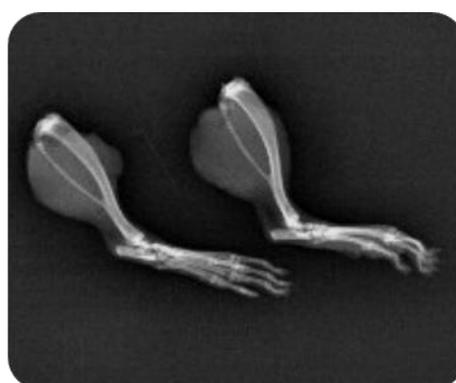

(A) Normal   **(B) Vehicle**

(C) Embelin (10mg/kg)   **(D) Embelin (30mg/kg)**

(E) Embelin (50mg/kg)   **(F) Prednisone (10mg/kg)**

Fig.6. Representative lateral X-ray image of the knee joint from after treatment with embelin

Table.1. Effect of Embelin on histopathology in joint of AA rats

Group	Dose (mg/kg)	Joint lesion score	
		Right Paw	**Left Paw**
Vehicle	-	4 (3-4)	3(2-4)
Embelin	10	3.5(3-4)	3(2-4)
	30	3.5(2-4)	2.5(2-4)
	50	3(2-4)*	2(1-3)*
Prednisone	10	3(3-4)	2(2-3)

Rats were immunized with Freund's complete adjuvant on right paw on day 0. Immunized rats were grouped randomly and treated with Embelin 10, 30 and 50 mg kg^{-1} day^{-1}, prednisone 10 mg kg^{-1} day^{-1} or placebo from day 19 to day 24; rats were sacrificed on day 29 and samples were collected for detection. Data expressed as median (minimum–maximum) by Mann–Whitney U-test; n = 10 rats for each group.*$P < 0.05$ vs. placebo-treated group, tested by Mann–Whitney U-test.

4. DISCUSSION

Rat adjuvant arthritis is an experimental model of polyarthritis which has been widely used for preclinical testing of numerous anti-arthritic agents which are either under preclinical or clinical investigation or are currently used as therapeutics in this disease. One of the most important features of AA is chronic synovitis, including inflammatory cell infiltration, pannus formation, cartilage destruction and bone erosion.[5] The hallmarks of this model are reliable onset and progression, at least 14–15 days of robust, easily measureable, polyarticular inflammation, marked bone resorption and periosteal bone proliferation. The appearance of polyarthritis initiates a secondary stage of the inflammatory response and the lesions in the paws can be seen by radiography on day 21 and day 25[12,13] In order to evaluate the therapeutic effect of Embelin on arthritis, Embelin was given to AA rats from day 19 to day 24. In the present study, we found that although the treatment stopped on day 24, the second inflammatory reactions and bone destructions were still inhibited by Embelin until the end of the experiment, especially in Embelin 50 mg kg^{-1} day^{-1} treated group. All these illustrated the therapeutic effect of Embelin on AA rats. Autoantigens that cross-react with Mycobacteria have been implicated in the pathogenesis of adjuvant arthritis in the rat.

Antibody to Mycobacterial antigen is reported in AA and also in rheumatoid arthritis. Adjuvant arthritis appears as a consequence of an immune response to cell wall of Mycobacterium. Compared with normal rats, AA rats had higher levels of IgG anti-Mycobacterium antibodies and the delayed skin reactions induced by the soluble fraction of Mycobacterium.[6] Embelin at dose 50 mg kg^{-1} day^{-1} significantly decreased humoral immune responses. At the same time, treatment with Embelin at all three doses assayed inhibited the delayed-type hypersensitivity seen in arthritic animals.[14] This work suggested that Embelin might exert its effect through its influence on the cellular and on the humoral immune response to Mycobacterium in adjuvant-induced arthritic rats. Based on the cytokine profile of RA, paracrine and autocrine networks may participate in disease perpetuation. Pro-inflammatory cytokines like interleukin-1β. and tumor necrosis factor alpha are important molecular mediators of immune and inflammatory responses.[15,16]

Excessive production of interleukin-1β and tumor necrosis factor-α is believed to contribute to the onset and progression of a number of inflammatory and autoimmune pathologies, such as rheumatoid arthritis and septic shock [17,18,5] Interleukin-1 and tumor necrosis factor-α promote induction of adhesion molecule, proteinase gene expression and play major role in

the progression of joint destruction and proliferation of synoviocytes. They can also increase production of interleukin-6, granulocyte-macrophage colony-stimulating factor, interleukin-8, and other chemokines.[19] These cytokines, in turn, can activate macrophages in the environment and lead to continued cytokine production. This creates and may contribute to pathophysiology of inflammatory processes in hind limb in the chronic phase.

In our study, AA rats expressed high levels of interleukin-1β, mRNA in synovial membrane and tumor necrosis factor-α mRNA in hind paw when compared with normal animals. The inhibitory effect of Embelin on interleukin-1β and tumor necrosis factor-α mRNA correlated with its effect on joint lesion reduction.[20]

In the light of the above results it might me concluded that the drug embelin exhibited a potent antiarthritic effect by reducing the pathological lesions via down regulating the levels of proinflammatory cytokines thereby reducing the levels of acute phase proteins and also via its enhanced immunomodulatory property on humoral and cellular immune responses. From the present study, we found that Embelin has therapeutic effect on AA, and the effect might relate to its suppressive effect on the production of pro-inflammatory cytokines and on humoral and cellular immune responses.

CONFLICT OF INTEREST STATEMENT
There is no conflict of interest.

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