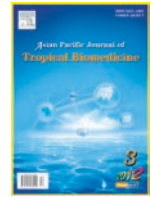




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## Evaluation of antiarthritic and immunomodulatory activity of *Barleria lupulina*

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## ABSTRACT

**Objective:** *Barleria lupulina* Lindl (Acanthaceae) (*B. lupulina*) has been traditionally used in the treatment of rheumatoid arthritis but, no scientific data has been published supporting the claimed ethnomedical use. This study was designed to investigate the anti–arthritic potential of *B. lupulina* leaves and its role in immunomodulation. **Methods:** Methanol extract of *B. lupulina* (MEBL) leaves (300 and 600 mg/kg BW) was tested for its antiarthritic activity by various models namely, formalin–induced arthritis, adjuvant induced arthritis, collagen type II–induced arthritis and monosodium iodoacetate induced osteoarthritis. Immunomodulatory activity of the same was tested by measuring WBC Count, Spleen Weight, Spleen WBC Count and Delayed Type Hypersensitivity (DTH) Reaction. **Results:** MEBL extracts 300 mg/kg and 600 mg/kg showed statistically significant inhibition ( $P < 0.05$  and  $P < 0.001$ ) of the edema formation and Myeloperoxidase (MPO) during experimental period and activities of antioxidants were restored significantly. MEBL extracts 300 mg/kg and 600 mg/kg significantly increased the Hemoglobin (Hb) level, serum albumin, total protein, calcium and phosphorus levels and reverted back the levels of WBC count and Erythrocyte Sedimentation Rate (ESR) ( $P < 0.05$  and  $P < 0.01$ ). Histopathological studies of ankle joints also supported this finding. Immunomodulatory study revealed an increase in the blood leukocytes count, weight of spleen, splenic leukocytes count and increase in paw volume on delayed type hypersensitivity footpad thickness suggesting an uplift of immune status. **Conclusions:** The present study concluded that, MEBL holds antiarthritic and Immunomodulatory activity. Although subsequent study is required to evaluate the active constituents responsible for the activity.

### 1. Introduction

Rheumatoid arthritis is a chronic inflammatory autoimmune disease, which is characterized by a series of pathological processes of the joints, such as leukocyte infiltration, pannus formation and extensive destruction of the cartilage and bone. Cartilage degradation is mediated by enzymes such as matrix metalloproteinase and glycosidase. Pro–inflammatory cytokines such as interleukin–1 (IL–1) and tumor necrosis factor (TNF), which are released during joint inflammation, stimulate production of these enzymes and promote cartilage degradation [1]. Though conventional treatment options for this condition have improved in terms of effectiveness, the use of non–steroidal anti–inflammatory drugs (NSAIDs) such as etoricoxib, disease modifying anti–rheumatic drugs (DMARDs) such as methotrexate,

sulphasalazine, leflunomide, hydroxychloroquine, and corticosteroids including prednisolone, methylprednisolone have all been associated with adverse effects [2]. Herbal medicines are being accepted and used increasingly by general populations in both eastern and western countries because of the ethnic acceptability and compatibility having fewer side effects [3]. This revival of herbal and other complementary therapies in the management of chronic diseases (RA and other inflammatory disorders) is well documented [2].

*Barleria lupulina* Lindl. (Acanthaceae) (*B. lupulina*) is a small herb, distributed in the South Asian region. It has been traditionally used for mental tension, diabetes, rheumatoid arthritis, and snake bite [4]. It has been reported as potent anti inflammatory agent [5,6]. Seven iridoid glucosides [7] and a betaine compound was isolated [8]. Several alkaloids were estimated from the stem and leaves of *B. lupulina* [8]. Antiviral activity against HSV–2 and anti–ulcer activity has been reported in various extracts of this plant [6]. *B. lupulina* has been traditionally used in the treatment of rheumatoid arthritis but no scientific data has been published

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supporting the claimed ethnomedical use. As rheumatoid arthritis is an autoimmune disorder, so it is expected that it must have the potential of immunomodulation. Hence this study is designed to investigate whether the acclaimed traditional use in rheumatoid arthritis of *B. lupulina* leaves has any scientific justification and also to evaluate its role in immunomodulation.

## 2. Materials and method

### 2.1. Plant Material

The leaves of *B. lupulina* were collected from B.I.T. Mesra, Ranchi in December 2009. The plant herbarium is authenticated by Dr. Tariq Husain, Scientist-in-charge, Herbarium, National Botanical Research Institute (Council of Scientific & Industrial Research), Lucknow (226001), India. The herbarium has been deposited in the Department of Pharmaceutical Sciences, Birla Institute of Technology Mesra, Ranchi.

### 2.2. Preparation of Extracts

The collected parts were carefully dried in shade for 15 d. To ensure complete dryness plants were kept in hot air oven at 45 °C for 5 min. Then, the powdered plants were stored at room temperature in an air tight container. The dried and powdered plant material (leaves) was subjected to hot extraction in a Soxhlet extraction apparatus with methanol. The average time period for extraction was 48 h. The extract was filtered and concentrated using the Rotary evaporator (Buchi US) and the extractive value was found to be 9.4% (w/w).

### 2.3. Animals

Animals were procured from Institutional Animal house of Birla Institute of Technology, Mesra. All animal experiments were strictly complied with the ethical standards of animal handling and approved by Institutional Animal Ethics Committee (621/02/ac/CPCSEA). The animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature 24–27 °C and humidity 60–65% with 12:12 light: dark cycles. Food was provided in the form of dry pellets (Hindustan Lever Ltd.) and water ad libitum. The animals were acclimatized under the laboratory conditions for 7 d before the commencement of experiment.

### 2.4. Preliminary phytoconstituents analysis

Preliminary tests were carried out for the presence or absence of phytoconstituents like Alkaloids, Carbohydrates, Flavonoids, Glycosides, Reducing sugars, Saponins, sterols, Anthocyanins, Terpenes and Tannins [9].

### 2.5. Treatment Protocol

Group 1 (vehicle control): treated with 2% (v/v) of Tween 80

at a dose of 10 ml/kg BW, i.p.

Group 2 (Standard): treated with Indomethacin at a dose of 10 mg/kg BW, i.p.

Group 3: treated with MEBL at a dose of 300 mg/Kg BW, i.p.

Group 4: treated with MEBL at a dose of 600 mg/Kg BW, i.p.

## 2.6. Antiarthritic activity

### 2.6.1. Formalin-induced arthritis

Albino male mice weighing between 25–35 g were used. They were divided into 4 groups with six mice in each group. All the treatments were given for a period of 10 days duration on the basis of treatment protocol. Formalin (0.1ml of 2% (w/v) solution in normal saline water) (Central Drug House (P) Ltd.) was injected in the sub plantar region of the right hind paw on 1st and 3rd day. Every day till 10th day paw volume of right hind paw was measured with the help of plethysmometer (IITC, Life science, Model 520) after respective treatments. The mean increase in the paw volume of both the groups over the period of 10 days was calculated and compared [10].

### 2.6.2. Adjuvant induced arthritis

Female Sprague Dawley rats weighing between 150–200 g were used. Animals were divided into 4 groups with six rats in each group. On Day 1, body weight and paw volumes of all the animals was measured. The animals (except normal control group) were immunized with complete Freund adjuvant. On appearance of the signs of arthritis (weight loss, increase in paw volume and swelling of joints), the respective treatment was started and continued till day 28. The mean difference of the paw volume was calculated and the significance was compared statistically with arthritic control (One-way ANOVA followed by Newman-Keul's Multiple Comparison test). Antioxidant parameters [11] viz. TBARS, Superoxide dismutase activity (SOD), glutathione, glutathione peroxidase were determined at 28th day. Myeloperoxidase activity [11] was also determined at 28th day.

### 2.6.3. Collagen type II-induced arthritis (CIA)

Albino male rats weighing between 150–200 g were used. Animals were divided into 4 groups with six rats in each group. CIA was induced in rats by multiple intradermal injections, at the base of the tail and into three to five other sites on the back, of 250 µg of chicken sternum type II collagen (Sigma) in 125 µL of 0.1 M acetic acid emulsified in an equal volume of complete Freund's adjuvant (Sigma) containing 2 mg/mL Mycobacterium tuberculosis. Rats were challenged again with the same antigen preparation 7 d later. Before injection, animals were anaesthetized with ether and injections were performed with a 15 gauge needle. Disease developed about 11–13 d after the second immunization. At day 14, animals were randomized to receive treatments according to the treatment protocol, timed to coincide approximately with the onset of arthritis pathology. All these doses administered daily, in a volume of 1 mL/kg body weight, intraperitoneally once a day until the 28th day. On 28th day animals were sacrificed and antioxidant parameters viz. TBARS, superoxide dismutase activity

(SOD), glutathione-S-transferase (GST) were determined. At the same day hematological parameters viz. leukocyte count, ESR, hemoglobin and biochemical parameters viz. albumin, total protein, calcium and phosphorus levels were determined. Then the histopathology of the ankle joints was done subsequently [12,13].

#### 2.6.4. Monosodium iodoacetate induced osteoarthritis

Albino Wistar strain rats weighing between 150–200 g were used. Animals were divided into 4 groups with six rats in each group. Animals were prepared for intra-articular injection of MIA (Sigma) by brief anaesthesia with ether. Intra-articular injection of 1 mg MIA in 20  $\mu$ L of 0.9% sterile saline (control 20  $\mu$ L of 0.9% sterile saline alone) was administered through the intra-patellar ligament into the joint space of the right knee via a glass syringe. Treatment was started 14 d after the MIA injection and it was continued till 28th day. Mechanical hyperalgesia was assessed by measuring withdrawal thresholds to calibrated Von Frey Apparatus (IITC, Life science) on 0th, 7th, 14th, 21st and 28th day [6]. On 28th day animals were sacrificed and biochemical parameters viz. albumin, calcium [14,15] and phosphorus [16] levels were determined. Then the histopathology of the ankle joints was done subsequently [17].

#### 2.7. Immunomodulatory activity

##### 2.7.1. Treatment Protocol

Male albino mice having 25–35 g body weight were used. Animals were divided into 3 groups with six rats in each group.

Group 1 (vehicle control): treated with 2% (v/v) of Tween 80 at a dose of 10 mL/kg, BW, i.p.

Group 2: treated with MEBL at a dose of 300 mg/kg BW, i.p.

Group 3: treated with MEBL at a dose of 600 mg/kg BW, i.p.

##### 2.7.2. WBC Count, spleen weight and spleen WBC count

All groups of mice were treated according to the treatment protocol for 5 d. On day 6, blood was collected from retro-orbital plexus for white blood cells (WBC) count. The animals were sacrificed by cervical dislocation and their spleens were harvested and weighed and the spleen leukocytes count was estimated. The results of these analyses were compared with that of control [18].

##### 2.7.3. Delayed type hypersensitivity (DTH) reaction

Animals of all group according to treatment protocol were sensitized with 10% Sheep Red blood cells (SRBC) at day 0 and day 7 subcutaneously (s.c.). Animals of group 2 and 3 were administered MEBL 300 mg/kg BW and 600 mg/kg BW, i.p. on days -4, -2, 0, 2, 4, 6, 8, whereas control group was administered with equal volume of 2% (v/v) Tween 80 solution. On day 9, groups 2 and 3 were challenged with 10% SRBC cells, intradermally into the left footpad of each mice, while Phosphate buffered saline (PBS) (pH 7.4) was injected into right hind paw. The increase in footpad thickness (FPT) was measured 24 h after SRBC challenge by plethysmometer (IITC, Life science, Model 520). The degree of DTH reaction was expressed as the percentage increase in FPT over the

control values [18].

#### 2.8. Statistical analysis

All the values were calculated as arithmetic mean  $\pm$  standard error of mean (SEM). The significance of the differences of the mean values with respect to control group was analyzed using one-way analysis of variance (ANOVA) followed by Dunnet's 't' test.

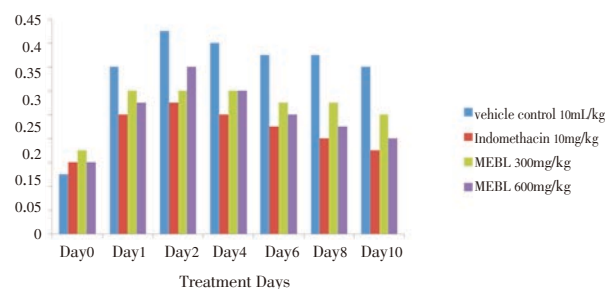
### 3. Results

#### 3.1. Preliminary phytoconstituents analysis

Preliminary tests of MEBL revealed the presence of phytoconstituents such as alkaloids, carbohydrates, flavonoids, glycosides, reducing sugars, saponins, sterols, terpenes and tannins.

#### 3.2. Formalin-induced arthritis

The results showed that MEBL at the both doses of 300 and 600 mg/kg BW showed significant [ $(P<0.05)$ ,  $(P<0.01)$ ] inhibition in the increase in paw volume of the right hind paw as compared to vehicle control group (Figure 1).



**Figure 1.** Effect of the MEBL on the Formalin induced mice right hind paw edema.

#### 3.3. Adjuvant induced arthritis

The results illustrated that MEBL at both the doses of 300 and 600 mg/kg BW showed significant inhibition [ $(P<0.05)$ ,  $(P<0.001)$ ] in the increase in paw volume of the both right (Figure 2) and left hind paws (Figure 3) as compared to vehicle control and arthritic control groups. MEBL at 300 mg/kg BW dose showed significant ( $P<0.05$ ) MPO inhibitory activity and at 600 mg/kg BW dose it showed highly significant ( $p<0.001$ ) MPO inhibitory activity that tantamount to standard drug indomethacin (10 mg/kg BW), as shown in the Table 1. Further table 1 also represents the extent of activities of enzymic (SOD and GPx) and of non-enzymic antioxidants (GSH) and TBARS in kidney tissue homogenate, respectively. The activities of all these antioxidants were found to be decreased in arthritic animals when compared to control animals. Upon treatment with MEBL at both the doses the antioxidant activity were restored significantly [ $(P<0.05)$ ,  $(P<0.01)$ ,  $(P<0.001)$ ] when compared to arthritic group.

**Table 1.**

Effect of MEBL on Myeloperoxidase inhibitory activity and Antioxidant level in the adjuvant induced rat.

Parameters	Normal control	Arthritic control	Standard drug10mg/kg BW	MEBL 300mg/kg BW	MEBL 600mg/kg BW
MPO Inhibitory activity	0.15±0.01	1.75±0.11	0.55±0.03c	1.60±0.04a	0.76±0.02c
SOD(U/ml)	8.72±0.14	3.03±0.19	5.93±0.13c	2.42±0.08b	3.62±0.12b
MDA(TBARS) nmol/mg of protein	5.15±0.32	13.83±0.6	7.66±0.42c	12.66±0.20a	8.32±0.21c
GSHpxase (U/ml)	41.15±1.22	18.83±0.83	31.15±0.4c	18.66±0.66	28.50±1.30c
GSH (U/ml)	6.70±0.14	2.13±0.23	4.48±0.16c	2.22±0.18c	3.32±0.06c

Values are reported as Mean±S.E.M. for six mice in each group  $aP < 0.05$ ;  $bP < 0.01$ ;  $cP < 0.001$  compared with controls using; a one-way analysis of variance (ANOVA) followed by Dunnett's t- test.

**Table 2.**

Effect of the leaves extracts of Barleria lupulina on the antioxidant level in the collagen type II induced rat.

Parameters	Vehicle control	Standard 10mg/kg BW	MEBL 300mg/kg BW	MEBL 600mg/kg BW
SOD(U/ml)	1.68±0.021	2.29±0.038**	1.72±0.022	1.85±0.017*
MDA(TBARS) nmol/mg of protein	6.76±0.375	3.66±0.088**	5.53±0.176*	4.83±0.240*
GST(U/ml)	0.79±0.023	0.97±0.067*	0.81±0.023	0.91±0.029
Albumin gm/dl	2.39±0.041	3.53±0.122**	2.87±0.0580.05774	3.20±0.220*
Calcium mg%	2.36±0.065	2.60±0.116**	3.50±0.042	3.43±0.203**
Phosphorus mg%	2.58±0.038	4.70±0.240**	2.87±0.038	3.10±0.110*
Totalprotein gm/dl	5.07±0.285	5.83±0.203*	6.17±0.203	6.40±0.265*

Values are reported as Mean±S.E.M. for six mice in each group  $*P < 0.05$ ;  $**P < 0.01$ ; compared with controls using; a one-way analysis of variance (ANOVA) followed by Dunnett's t- test.

**Table 3.**

Effect of the leaves extracts of Barleria lupulina on the hematological parameters in the collagen type II induced rat.

Parameters	Vehicle control	Standard 10mg/kg BW	MEBL 300mg/kg BW	MEBL 600mg/kg BW
Leukocyte count Cells/cmm	10500±288.6	7000±305.5b	9600±208.2	9266.67±120.2a
ESR	30 min	6.2±0.4	3.20±0.0b	5.15±0.1a
		60 min	8.0±0.4	3.95±0.2 b
Hb (gm/dl)	10.1±0.3	12.12±0.9b	12.3±0.7b	10.23±0.3

Values are reported as Mean±S.E.M. for six mice in each group  $aP < 0.05$ ;  $bP < 0.01$ ; compared with controls using; a one-way analysis of variance (ANOVA) followed by Dunnett's t- test.

**Table 4.**

Effect of the leaves extracts of Barleria lupulina on the biochemical parameters in the MIA induced rat.

Parameters	Normal control	Arthritic control	MEBL 300mg/kg BW	MEBL 600mg/kg BW
Albumin	3.75±0.25	3.35± 0.15	3.45±0.05	3.55± 0.05
Calcium	4.52±0.22	2.54±0.06	2.58±0.09*	3.62±0.18**
Phosphorus	4.92±0.17	2.97±0.09	2.93±0.14*	3.89± 0.19**

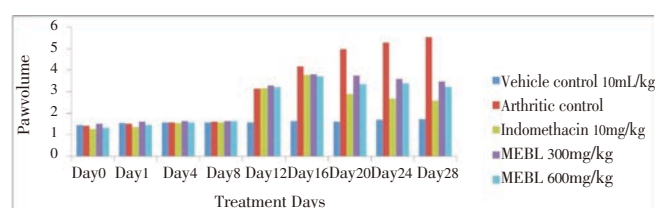
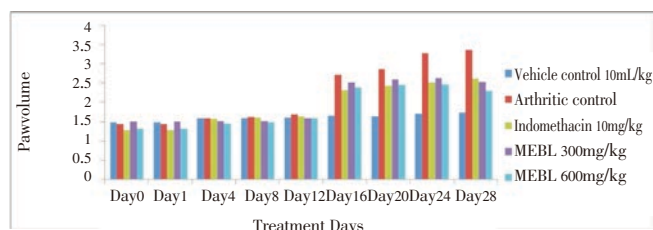
Values are reported as Mean±S.E.M. for six mice in each group  $*P < 0.05$ ;  $**P < 0.01$ ; compared with controls using; a one-way analysis of variance (ANOVA) followed by Dunnett's t- test.

**Table 5.**

Effect of MEBL on spleen weight, WBC count spleen leukocyte count and percentage increase in paw volume

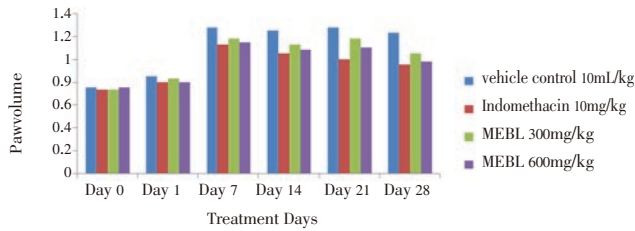
Treatment groups	Spleen weight (gm)/100gm BW	WBC count (cells/cmm)	Spleen leukocyte count (cells/cmm)	Percentage increase in paw volume
control	0.115±0.005457	4400±113.65	33833.33±1965.0	23.39±0.6589
300mg/kg BW	0.142±0.003844*	4850±225.46	46333.33±2962.7*	26.06±0.7839*
600mg/kg BW	0.205±0.005774**	5125±125*	92666.67±1453.0**	33.4±0.4933**

Values are expressed as Mean ±SEM. \*  $P < 0.05$ , \*\* $P < 0.01$  (compared to respective control).

**Figure 2.** Effect of the MEBL on the adjuvant induced rat right hind paw edema.**Figure 3.** Effect of MEBL on the adjuvant induced rat left hind paw edema.

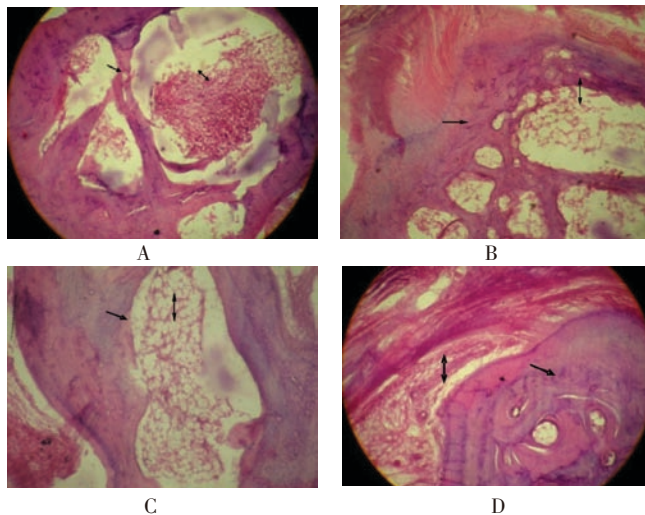
### 3.4. Collagen type II-induced arthritis in rats

The results revealed that MEBL at its both doses 300 and 600 mg/ kg BW showed significant inhibition [( $P < 0.05$ ), ( $P < 0.01$ )] respectively in the increase in paw volume of the right hind paw as compared to vehicle control (Fig 4).



**Figure 4.** Effect of the leaves extracts of *Barleria lupulina* on the collagen type II induced rat left hind paw edema.

Table 2 represents the extent of activities of enzymic (SOD and GST) antioxidants and TBARS levels in kidney tissue homogenate, respectively. The activity of SOD was found to be significantly [( $P < 0.05$ ), ( $P < 0.01$ )] increased in treated animals as compared to the arthritic animals where as the activity of TBARS was found to be significantly [( $P < 0.05$ ), ( $P < 0.01$ )] decreased upon treatment with MEBL but there was no significant ( $P > 0.05$ ) effect of this extract on GST level. In addition, Table 2 represents the biochemical changes



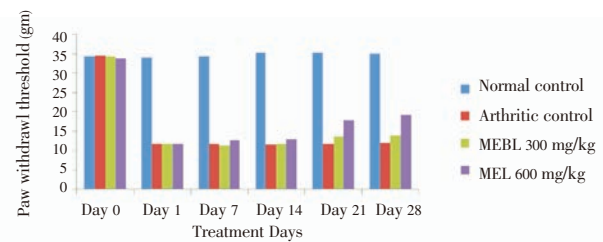
**Figure 5.** Histological changes in ankle joints of collagen type II induced rat.

(A) Histological changes in ankle joints of arthritic control animal. Section of joint cavity showing severe periarticular inflammation with marked edema is present with areas of bone resorption (double arrow) as well as the damaged articular cartilage (single arrow) (B) Histological changes in ankle joints of animal treated with MEBL 300mg/kg BW, i.p. Periarticular inflammation is there but it is less than the arthritic control (double arrow is showing the periarticular inflammation along with edema). Damaged articular cartilage is repairing (single arrow). (C) Histological changes in ankle joints of animal treated with MEBL 600mg/kg BW, i.p. Periarticular inflammation is gradually recovering (double arrow is showing the periarticular inflammation along with less edema). Articular cartilage is repairing (single arrow). (D) Histological changes in ankle joints of animal treated with standard drug indomethacin 10mg/kg BW, i.p. Periarticular inflammation is very less double arrow is presenting the position. Articular cartilage is largely unaffected.

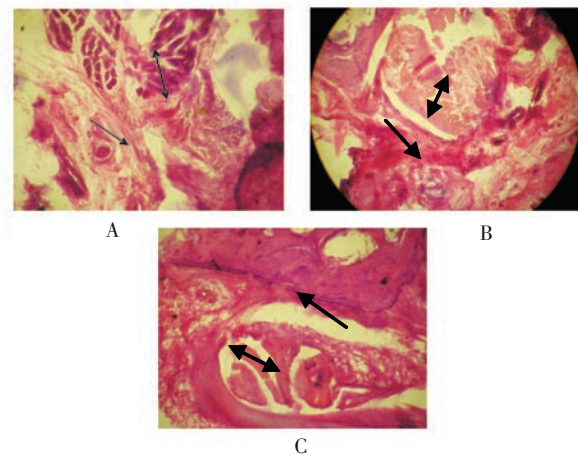
associated with arthritic condition. Albumin, total protein, calcium and phosphorus levels were significantly [( $P < 0.05$ ), ( $P < 0.01$ )] increased upon treatment with extract as compared to the vehicle control group. Table 3 represents the hematological changes associated with arthritic condition. Levels of Haemoglobin (Hb) were increased significantly ( $P < 0.01$ ) on treatment with MEBL 300 mg/ kg BW, and standard groups. WBC count and ESR were reverted to near normal levels in MEBL treated animals that is comparable to the standard drug (indomethacin 10 mg/ kg BW). The ankle joint of the hind paw of the rats were removed and separated from the surrounding tissues and weighed. The joints fixed in 10% formalin were decalcified, sectioned and finally stained with haematoxylin and eosin. Histopathological changes in all the above groups were recorded (Fig 5).

### 3.5. Monosodium iodoacetate induced osteoarthritis

It was found that MEBL at its both doses of 300 and 600 mg/ kg BW showed significant increase [( $P < 0.05$ ), ( $P < 0.01$ )] respectively in paw withdrawal threshold (g) as compared to arthritic control on 21st and 28th day (Fig 6). Biochemical parameters viz. serum albumin, calcium and phosphorus concentration were assessed and the results were tabulated in Table 4. It was found that albumin concentration did not



**Figure 6.** Effect of the leaves extracts of *Barleria lupulina* on the paw withdrawal threshold (gm) in MIA induced rats.



**Figure 7.** (A) Histological changes in ankle joint of arthritic control animal. Section of joint cavity showing profound loss of articular cartilage and subchondral bone with ongoing loss of epiphyseal cartilage and bone tissue. (B) Histological changes in ankle joints of animal treated with MEBL 300 mg/kg BW, i.p. Section of joint cavity showing that loss of articular cartilage and subchondral bone with ongoing loss of epiphyseal cartilage and bone tissue was comparatively less than the arthritic control. (C) Histological changes in ankle joints of animal treated with MEBL 600 mg/kg BW, i.p. Section of joint cavity showing that loss of articular cartilage and subchondral bone with ongoing loss of epiphyseal cartilage and bone tissue was comparatively less than the animal treated with MEBL 300 mg/kg BW, i.p.

change significantly ( $P>0.05$ ) in arthritic control animals as compared to normal control whereas calcium and phosphorus concentration decreased significantly ( $p<0.05$ ) in the arthritic control animals as compared to the normal control group. Calcium and phosphorus concentration were improved significantly ( $P<0.01$ ) upon treatment with MEHL 600 mg/ kg BW. Fig 7 represents the histological changes associated with the ankle joint of the arthritic and the treated (MEHL 300 mg/ kg BW, MEHL 600 mg/ kg BW) animals.

### 3.6. Immunomodulatory activity

The effect of MEHL 300 mg/ Kg BW, 600 mg/ kg BW on spleen weight, WBC count, spleen leukocyte count and percentage increase in paw volume on delayed type hypersensitivity footpad thickness is shown in Table 5. MEHL 600 mg/ kg BW showed positive effect ( $P<0.01$ ) in all parameters.

## 4. Discussion

The acute stage of arthritis is characterized by the signs of hyperalgesia, lack of mobility and pause in body weight gain (data not shown). During the acute period, hind paw and fore paw joint diameters increased. In the formaldehyde induced arthritis inflammation test, MEHL extracts showed significant inhibition of the edema formation during experimental period of 10 days. In the later acute stages of the disease (day 12 onwards) rats with adjuvant arthritis were relatively immobile due to the severity of paw swelling.

It was found that MEHL significantly inhibited the development of chronic swelling induced by complete Freund's adjuvant, and showed significant MPO inhibitory activity at both the doses. The activities of some antioxidants (SOD, GSH, MDA and GSHpxase) were found to be decreased in arthritic animals when compared to control animals. Upon treatment with MEHL at both the doses the activities of antioxidants were restored significantly.

In the collagen type II induced arthritis, decrease in Hb level and increase in WBC count and ESR was observed. Decrease in Hb level reflects the presence of anemia in arthritic rats. Anemia is the most common extra cellular manifestation of RA. The increase in WBC might be due to the stimulation of immune system against the invading pathogenic microorganism. ESR is an indirect measurement of acute phase response for determining the disease activity in RA. The above-mentioned changes were brought back to near normal levels upon MEHL treatments, which emphasized the beneficial effect of the drugs on CIA. In the collagen type II induced arthritis, decrease in antioxidants (SOD, TBARS and GST) levels were observed which was reverted to near normal on treatment with MEHL. In the collagen type II induced arthritis decrease in serum albumin, total protein, calcium and phosphorus levels were also observed. Hypoalbuminaemia is the characteristics of the chronic inflammatory disease. Loss of bone and degradation of the cartilage is one the most important features of the RA. Thus here decreased level of Calcium and Phosphorus representing the bone and cartilage loss in CIA. The above-mentioned changes were brought back to near normal levels upon MEHL treatments, which again emphasizes the

beneficial effect of the drugs on CIA. These findings seem to justify the use of the plant in traditional medicine in the treatment RA.

Epidemiological studies have revealed that over 70% of people aged 65 years or older suffer from osteoarthritis (OA) with the knee joint being most commonly affected [19]. Despite the widespread prevalence of OA in the adult population, very little is known about the causes of OA pain or the chemical mediators involved in the initiation of painful stimuli in OA joints. Iodoacetate injection in the ankle joint of the rat induced histological changes and pain related behaviours characteristic of human OA. Although the behavioral changes and histology both worsened over time, the majority of the pain responses were apparent within few moment of the iodoacetate injection, whereas gross joint damage was not evident until around day 21. Cartilage degradation is perhaps the best-studied feature of this model.

The anatomical features that show an association with joint pain are osteophytes [20], bone edema [19] and synovitis [21]. However, these findings have not all been confirmed by other investigators [22]. The present investigation found that intra-articular injection of MIA caused mechanical hyperalgesia in right hind paw. Plantar analgesimetry experiments demonstrated that the force required to elicit hind paw withdrawal threshold was significantly ( $P<0.05$ ) reduced in arthritic control animals as compared to the normal control.

Again this PWT were significantly [ $(P<0.05)$ ,  $(P<0.01)$ ] increase in the treated (MEHL 300mg/kg BW, MEHL 600mg/kg BW) groups respectively as compared to arthritic control on 21st and 28th day. Hence it is provocative to speculate that the methanol extract of *Barleria lupulina* leaves is showing some beneficial effect on behavioral pain response. The decrease in serum calcium and phosphorus concentration in arthritic control animals is indicative of loss of cartilage and bone.

Among different organs of immune system, spleen represents a major secondary lymphoid organ involved in elicitation of immune response. Unlike lymph nodes, which are specialized to trap-localized antigen from regional tissue spaces, the spleen is adapted to filtering blood and trapping blood-borne antigens and thus can respond to systemic infections [23, 24]. Results revealed an increase in the blood leukocytes count, weight of spleen and splenic leukocytes count suggesting an uplift of immune status.

Delayed type hypersensitivity (DTH) reaction is characterized by large influxes of non-specific inflammatory cells, in which the macrophage is a major participant. It is a type IV hypersensitivity reaction that develops when antigen activates sensitized TDTH cells [delayed type hypersensitivity (T-Cells)]. These cells generally appear to be a TH1 subpopulation although sometimes TC cells are also involved. Activation of TDTH cells by antigen presented through appropriate antigen presenting cells results in the secretion of various cytokines including interleukin-2, interferon- $\alpha$ , macrophage migration inhibition factor and tumor necrosis factor [25]. The overall effects of these cytokines are to recruits macrophages into the area and activate them, promoting increased phagocytic activity vis-a-vis increased concentration of lytic enzymes for more effective killing. Several lines of evidence suggest that

DTH reaction is important in host defense against parasites and bacteria that can live and proliferate intracellularly. Treatment of MEBL enhanced DTH reaction, which is reflected from the increased footpad thickness compared to control group suggesting heightened infiltration of macrophages to the inflammatory site. This study may be supporting a possible role of MEBL in assisting cell-mediated immune response.

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### Conflict of interest statement

We declare that we have no conflict of interest.

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### Conflict of interest statement

The authors report no declaration of interests. The authors alone are responsible for the content and writing of the paper.

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