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Anti-arthritic effect of β-caryophyllene and its ameliorative role on methotrexate and/or leflunomide-induced side effects in arthritic rats

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ABSTRACT

Aim: Rheumatoid arthritis (RA) is the most widespread inflammatory arthropathy, which causes severe disability. It is highly important to ameliorate the side effects caused by different drugs used to treat RA. Therefore, this study assessed the potential role of β-caryophyllene (BCP) in treating adjuvant-induced arthritis (AIA), increasing the efficacy of methotrexate (MTX) and/or leflunomide (LEF), and ameliorating their side effects.

Material and methods: AIA was induced in rats by injecting complete Freund's adjuvant. The rats were divided into different groups such as sham group; control group; monotherapy groups, including BCP (300 mg/kg), MTX (1 mg/kg), and LEF (10 mg/kg); and combined groups, including MTX + BCP, LEF + BCP, MTX + LEF, and MTX + LEF + BCP groups.

Key findings: Monotherapy with BCP or MTX or LEF as well as MTX + LEF significantly reduced paw thickness and arthritic index; the histopathological changes in hind paw joints were recovered; and oxidative stress and tumor necrosis factor-alpha (TNF-α) levels in arthritic rats were reduced. The co-administration of BCP and MTX and/or LEF significantly improved the therapeutic efficacy of MTX and/or LEF and significantly reduced the myelosuppressive and hepatotoxic effects of MTX and/or LEF. Taken together, BCP could be used with MTX and/or LEF for the treatment of RA to reduce the side effects of the drugs and increase their efficacy.

1. Introduction

Rheumatoid arthritis (RA) is a long-standing, complex, multifarious, and prevalent autoimmune disease (AD) and it is accountable for remarkable morbidity. In an AD such as RA, the immune system, which normally protects the body, erroneously attacks the tissues and joints. It is characterized by chronic inflammation of the diarthrodial joints leading to symmetrical polyarthritis and synovial membrane hypertrophy with advanced destruction and distortion of the joints, bones, and cartilages [1,2]. The immune cells, mainly T cells and B cells, and the organized interaction of pro-inflammatory cytokines play critical roles in RA pathogenesis. The production of pro-inflammatory cytokines, such as TNF-α and IL-6, induces synovial inflammation [3]. In addition to inflammation, oxidative stress also rises in the joints and periphery [4].

Disease-modifying anti-rheumatic drugs (DMARDs) are approved and frequently used to treat RA [5]. These immunosuppressive drugs slow down joint damage and inhibit morbidity in the long term. Methotrexate (MTX) is the first approved and the most commonly used DMARD for RA patients [6]. MTX therapy slows down the rate of joint destruction and enhances the patient's quality of life. MTX should be combined with other drugs if MTX monotherapy does not provide sufficient healing. Leflunomide (LEF) is a popular medicine used in combination with MTX [7]. Although combination therapy provides better therapeutic benefits [8], increased myelosuppressive and hepatotoxic effects have been recorded [9]. Therefore, for the treatment of RA, we need effective drugs without toxicity.

Growing evidence demonstrates that the endocannabinoid system (ECS), particularly cannabinoid *receptor* type 2 (CB2), plays a key role in RA pathophysiology. Several members of ECS have been documented

Abbreviations: AIA, adjuvant induced arthritis; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BCP, β-caryophyllene; GSH, glutathione; LEF, leflunomide; MDA, malondialdehyde; MTX, methotrexate; RA, rheumatoid arthritis; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α

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to decrease RA synovial inflammation, hyperplasia, and cartilage damage. CB2 activation may ameliorate RA by suppressing the production of pro-inflammatory cytokines, autoantibodies, and matrix metalloproteinases (MMPs). This also inhibits bone deterioration, T cell-mediated immune response, and fibroblast-like synoviocyte (FLSs) proliferation [10]. Many selective CB2 receptor agonists provided beneficial effects in arthritis by reducing pain and modulating immune-inflammatory responses [11]. β-caryophyllene (BCP), a selective CB2 activator, is a bicyclic sesquiterpene detected in essential oils of cloves, basil, oregano, thyme, cinnamon, black pepper, *Copaifera* spp., and *Cannabis* spp. [12,13]. This compound seems to be interesting in drug development. Therefore, this study assessed the potential role of BCP in treating adjuvant-induced arthritis (AIA), increasing the efficacy of MTX and/or LEF in the treatment of AIA, and ameliorating their side effects.

2. Material and methods

2.1. Chemicals and drugs

Complete Freund's adjuvant (CFA) and BCP were obtained from Sigma-Aldrich (St Louis, MO, USA). MTX, a yellow crystalline powder, is manufactured by Shanxi PUDE Pharmaceutical Co., Ltd. LEF (Arava), as 10 mg, tablets is produced by Aventis Chemical Co.

2.2. Experimental animals

Experiments were conducted on adult male Wistar rats weighing 180–200 g ($n = 90$) obtained from the animal house at the Faculty of Veterinary Medicine, Zagazig University. The rats were housed in metal cages with a 12 h light cycle at 23 ± 2 °C and 40%–60% relative humidity. Rats were fed on an ad libitum diet throughout the experiment. The rats were adapted to the experimental location for two weeks prior to testing. Animal housing and care and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Zagazig University, Egypt (ZU-IACUC/2/F/47/2019).

2.3. Induction of AIA and experimental protocol

The rats were weighed and randomly allocated to nine groups consisting of ten animals per group ($n = 10$). The rats in group I (vehicle control group) were subcutaneously injected with 0.25 mL of paraffin oil into the palmar surface of the left hind paw and orally administered with corn oil. In all other groups, AIA was induced by subcutaneously injecting 0.25 mL of CFA (1 mL of CFA contained 1 mg of heat-killed and dried *Mycobacterium tuberculosis* (H37Ra, ATCC 25177) suspended in paraffin oil) in the palmar surface of the left hind paw of the rats [9,14].

Four days after inducing arthritis, various drug regimens were initiated that continued for four weeks. Further in group II (AIA group), arthritic rats were orally administered with corn oil; group III (BCP), arthritic rats were orally administered with BCP 300 mg/kg/day in corn oil [15]; group IV (MTX), arthritic rats were injected intraperitoneally MTX 1 mg/kg/week [16]; group V (MTX + BCP), arthritic rats were co-treated with MTX and BCP; group VI (LEF), arthritic rats were orally administered with LEF 10 mg/kg/day [17]; group VII (LEF + BCP), arthritic rats co-administered with LEF and BCP; group VIII (MTX + LEF), arthritic rats, were co-administered with LEF and MTX; and group IX (MTX + LEF + BCP), arthritic rats were co-administered with LEF, MTX, and BCP. The rats in all groups were carefully observed throughout the study.

2.4. Assessment of arthritis progression

The anteroposterior diameter of the inflamed left hind paw was measured using a Vernier caliper [18]. The severity of arthritis was evaluated by using a macroscopic scoring system as follows; 0, no swelling and erythema; 1, slight swelling and/or erythema; 2, mild swelling and erythema; 3, pronounced swelling and erythema with limited joint usage; and 4, gross deformity and disability to use limb [15]. Paw diameter was measured and the scoring of arthritis severity was done on 0, 4, 11, 18, 25, and 32 days after CFA injection.

2.5. Sampling

After 32 days, overnight fasted rats were carefully weighed and anesthetized by intramuscularly injecting ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg) mixture. Blood samples were collected from retro-orbital venous plexus with a fine sterilized glass capillary tube. Two different blood samples were collected from each rat; 0.5 mL in EDTA tube for hematological assessment and 2 mL in a glass tube without EDTA. These samples were left at room temperature for 10 min to coagulate to prepare serum. Sera samples were kept at −80 °C until further use. The rats were euthanized by decapitation, and liver, spleen, hind paw joint, and femur bone were rapidly dissected and stored in 10% neutral buffered formalin for histopathological examination.

2.6. Evaluation of hematological and biochemical parameters

Blood with EDTA was used to estimate complete blood cell count using a 902 automatic analyzer (HITACHI, Roche). Preserved serum was used to determine reduced glutathione (GSH), malondialdehyde (MDA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) [19–22]. Serum TNF-α level was measured using a specific rat ELISA kit provided by MyBioSource (San Diego, California, USA) according to the manufacturer's protocol.

2.7. Histopathological investigations

The specimens of hind paw joint and femur fixed in 10% neutral buffered formalin were first decalcified using equal parts of formic acid (8%) and hydrochloric acid (8%) with daily changes for four days to ensure proper softening of the tissue [23]. The specimens of hind paw joint, midshaft femur, spleen, and liver were processed, sectioned (5 μm thickness), and stained with hematoxylin and eosin [24]. Photomicrographs were taken with a digital camera (Canon) connected to a light microscope (Zeiss).

2.8. Morphometric analysis

For histoplanimetry analysis, ten rats were evaluated per group and three non-overlapping hematoxylin and eosin-stained sections of each rat per group were used. The histological lesion scores for paw joints were evaluated (×100 magnification) based on synovial membrane proliferation, pannus formation, and articular cartilage erosion. The scoring was as follows: 0, normal paw joints; 1, paw joints with few flat to round synovial lining cells with minimal infiltration of inflammatory cells, minimal pannus formation in marginal zones with few destructed cartilage; 2, paws with many flat to round synovial lining cells with mild infiltration of inflammatory cells, mild pannus formation with focal cartilage erosion; 3, paws with synoviocytes proliferated over three layers, moderate pannus formation with moderate cartilage erosion; 4, marked synovitis, severe pannus formation over joint surface with marked erosion of articular cartilages and subchondral bones [25]. Using digital images of the spleen (×100 magnification; Image J software), we calculated the ratio (%) of splenic white pulp to red pulp by dividing white pulp or red pulp area by the total splenic area and then multiplying by 100. Using digital images of the bone marrow (×400 magnification; Image J software (ver. 1.32j, http://rsb.info.nih.gov/ij)),

The ratio (%) of hematopoietic and fat tissues areas

$$= \frac{\text{Areas of the hematopoietic or fat tissue}}{\text{The field area}} \times 100$$

Hepatic lesion score (0 to 4) was assigned by portal inflammation, portal fibrosis, focal necrosis, and focal inflammation from liver images (×100 magnification) as follows: Portal inflammation: (0- absence, 1- mild, 2- moderate, 3- marked, 4- severe inflammation); portal fibrosis (0- absence, 1- mild, 2- moderate, 3- marked, 4- severe fibrosis); focal necrosis and focal inflammation (0- absence, 1- one or less focus, 2- two to four foci, 3- five to ten foci, 4- more than ten foci) [26].

2.9. Statistical analysis

Data presented are mean ± SE. All statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple-range post-hoc comparison test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of β-caryophyllene on the therapeutic efficacy of methotrexate and/or leflunomide

3.1.1. Paw thickness

Subcutaneous administration of CFA significantly ($p < 0.05$) increased hind paw thickness and arthritic index from 4th to 32nd day post-injection in AIA group compared with vehicle control group. Monotherapy with BCP or MTX or LEF as well as the combination therapy significantly ($p < 0.05$) reduced paw thickness and arthritic index compared with AIA group throughout the experimental period. Combination therapy (MTX + LEF) significantly ($p < 0.05$) reduced paw thickness and arthritic index compared with MTX or LEF alone. Further, co-administration of BCP and MTX or LEF or MTX + LEF significantly ($p < 0.05$) reduced paw thickness and arthritic index when compared with MTX or LEF or MTX + LEF (Fig. 1).

3.1.2. Serum TNF-α level and oxidant/antioxidant status

The AIA group showed significant ($p < 0.05$) increase in serum TNF-α and MDA levels and a significant ($p < 0.05$) reduction in serum GSH level compared with the vehicle control group. Monotherapy with BCP or MTX or LEF induced significant ($p < 0.05$) reduction in serum TNF-α and MDA levels and a significant increase in serum GSH level compared with the AIA group. The combination therapy (MTX + LEF) showed a significant reduction in the serum MDA level (19.08 nmol/mL) compared with LEF monotherapy (32.4 nmol/mL). Meanwhile, co-administration of BCP and MTX or LEF or MTX + LEF induced significant ($p < 0.05$) reduction in serum TNF-α and MDA levels and a significant increase in serum GSH level compared with the MTX or LEF or MTX + LEF group (Table 1).

3.1.3. Architecture of paw joint

Paw joint architecture was normal with a smooth surface of articular cartilage, subchondral bone, and monolayer synovial membrane in the vehicle control group. Subcutaneous injection of CFA in the hind paw disrupted the articular surface with massive degenerative and destructive changes in the cartilage and bone. In addition, we noticed severe synovial pannus formation with hypervascularization, marked hyperplasia of the synovial membrane, and cellular infiltration. In arthritic the rats treated with BCP or MTX or LEF, the articular surface exhibited only focal erosion of articular cartilage with moderate synovial pannus formation, moderate hyperplasia of the synovial membrane, and cellular infiltration. However, MTX + BCP and LEF + BCP groups exhibited nearly normal articular surface of only small focal erosion of articular cartilage with mild synovial membrane proliferation and cellular infiltration. In the MTX + LEF group, we observed superficial notched cartilage with a thin synovial membrane. Interestingly, the normal smooth surface of articular cartilage and subchondral bone with a thin synovial membrane were restored in the MTX + LEF + BCP group (Fig. 2A). Histological lesion score of paw joints corroborated these findings (Fig. 2B).

3.2. Effect of β-caryophyllene on the adverse effects of methotrexate and/or leflunomide

3.2.1. Hematological parameters

The AIA group showed a significant ($p < 0.05$) decrease in RBC count, Hb concentration, PCV, monocytes, eosinophils, and neutrophils

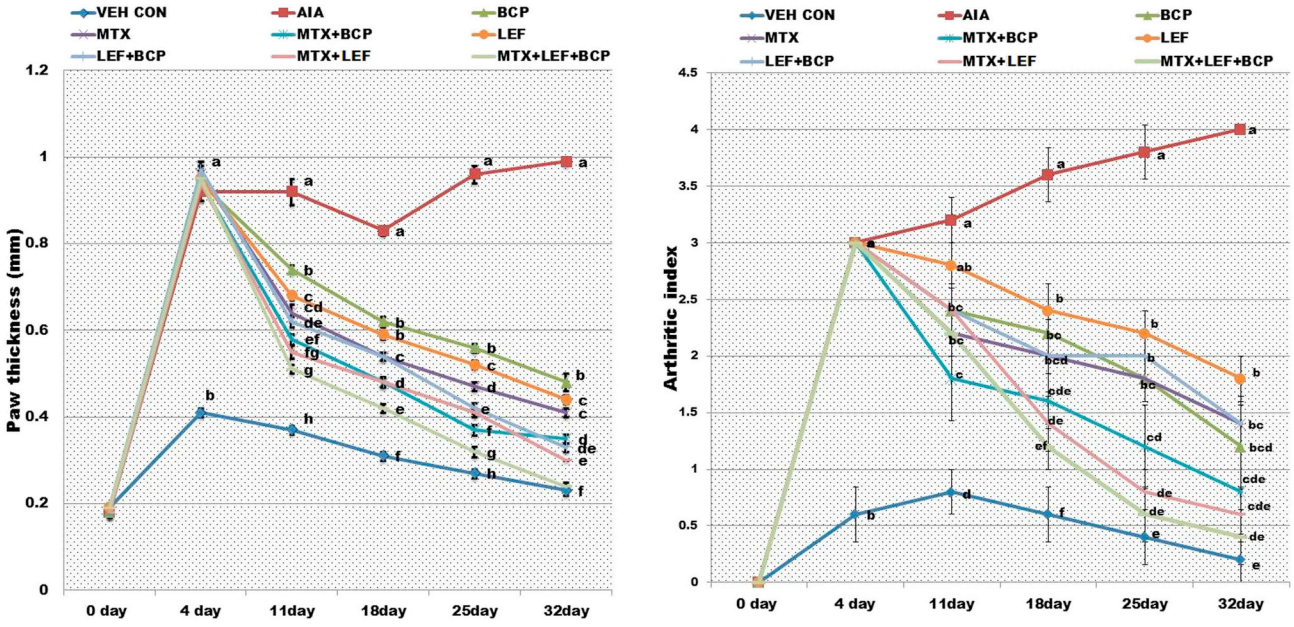

Fig. 1. Effects of oral administration of β-caryophyllene on paw thickness and arthritic index of AIA rats treated with methotrexate and/or leflunomide. The values are represented as the mean ± SE. In the same day, bars carrying different superscripts letters (a, b, c, d, e, f, g, h) are significantly different as analyzed by one-way ANOVA, followed by the multiple comparisons by Duncan's Post-hoc test ($p < 0.05$); $n = 10$ in each experimental group.

Table 1
Effects of oral administration of β-caryophyllene on the biochemical parameters of AIA rats treated with methotrexate and/or leflunomide.

Parameters	Groups								
	Vehicle control	AIA	BCP	MTX	MTX + BCP	LEF	LEF + BCP	MTX + LEF	MTX + LEF + BCP
TNF-α (pg/mL)	8.9 ± 0.73^{f}	30 ± 1.58^{a}	16.6 ± 1.2^{bcd}	14.6 ± 1.2^{cd}	10 ± 0.18^{ef}	20 ± 2.08^{b}	15.2 ± 2.16^{cd}	18 ± 0.57^{bc}	13.4 ± 0.45^{de}
GSH (μmol/mL)	1.2 ± 0.06^{a}	0.59 ± 0.01^{f}	0.9 ± 0.06^{b}	0.70 ± 0.01^{e}	0.80 ± 0.03^{cd}	0.74 ± 0.01^{de}	0.91 ± 0.01^{bc}	0.69 ± 0.002^{e}	0.82 ± 0.02^{bcd}
MDA (nmol/mL)	9.4 ± 0.748^{f}	45.6 ± 3.6^{a}	21.2 ± 1.4^{cd}	23.4 ± 1.66^{c}	16.2 ± 0.86^{de}	32.4 ± 2.78^{b}	21.9 ± 1.3^{c}	19.08 ± 0.75^{cd}	13 ± 0.3^{ef}
ALT (U/L)	51.8 ± 1.46^{g}	62.6 ± 1.3^{ef}	54.4 ± 1.02^{g}	69.38 ± 0.68^{d}	59.2 ± 0.73^{f}	73.8 ± 1.8^{c}	64.9 ± 1.4^{e}	92.2 ± 2.26^{a}	80.6 ± 1.12^{b}
AST (U/L)	208.8 ± 2.8^{h}	228.4 ± 1.8^{fg}	217.2 ± 3^{gh}	252 ± 4.3^{d}	232 ± 3^{ef}	279.6 ± 7.5^{c}	245.4 ± 5.9^{de}	330.2 ± 5.2^{a}	300.8 ± 6.6^{b}
ALP (IU/L)	222.8 ± 2.9^{f}	246.8 ± 6.2^{e}	231.2 ± 2.7^{f}	336.6 ± 3.2^{c}	317 ± 4.8^{d}	338 ± 8.92^{c}	310.2 ± 8.2^{d}	375.8 ± 2.6^{a}	355.6 ± 3.4^{b}

Values are represented as the mean ± SE (n = 10). Vehicle control: subcutaneously injected with 0.25 mL of paraffin oil into the palmar surface of the left hind paw and orally received corn oil; AIA: subcutaneously injected with 0.25 mL of CFA and orally received corn oil; BCP: subcutaneously injected with 0.25 mL of CFA and orally received 300 mg BCP/kg/day in corn oil; MTX: subcutaneously injected with 0.25 mL of CFA and IP injected with 1 mg MTX/kg/weekly; MTX + BCP: subcutaneously injected with 0.25 mL of CFA and co-treated with MTX and BCP; LEF, subcutaneously injected with 0.25 mL of CFA and orally received 10 mg LEF/kg/day; LEF + BCP: subcutaneously injected with 0.25 mL of CFA and co-administered with LEF and BCP; MTX + LEF: subcutaneously injected with 0.25 mL of CFA and co-treated with LEF and MTX and MTX + LEF + BCP: subcutaneously injected with 0.25 mL of CFA and co-administered with LEF, MTX and BCP. All treatments continued for four weeks. Mean values for the same parameter carrying different superscripts (a, b, c, d, e, f, g, h) are significantly different at $p < 0.05$. TNF-α: Tumor necrosis factor-alpha. GSH: Reduced glutathione. MDA: Malondialdehyde. ALT: Alanine Aminotransferase. AST: Aspartate transaminase. ALP: Alkaline phosphatase.

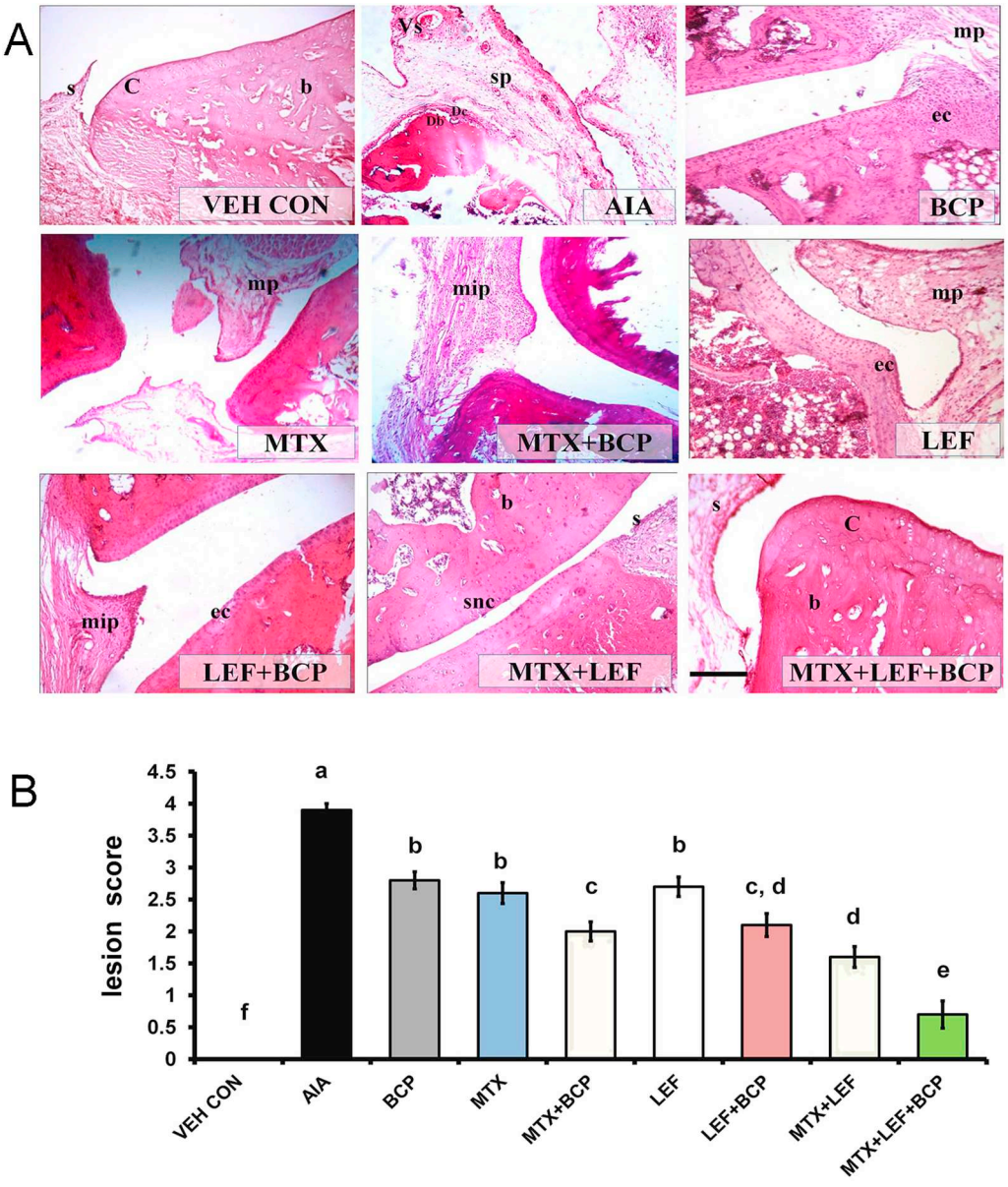

Fig. 2. Evaluation of the changes in the hind paw joints of the rates in the different studied groups. (A) Representative photomicrographs from vehicle control (VEH CON), adjuvant-induced arthritis (AIA), β-caryophyllene (BCP), methotrexate (MTX), methotrexate and β-caryophyllene (MTX + BCP), leflunomide (LEF), leflunomide and β-caryophyllene (LEF + BCP), methotrexate and leflunomide (MTX + LEF), methotrexate, leflunomide and β-caryophyllene (MTX + LEF + BCP) groups showing the normal joint structure with normal articular cartilage (C), subchondral bone (b), monolayer synovial membrane (s). The synovial pannus (sp) of obvious cellular infiltration, hypervascularization (Vs) and synovial membrane hyperplasia over the articular surface of the degenerated bone (Db) and cartilaginous (Dc) tissues were observed. Moderate synovial membrane proliferation (mp) with focal eroded articular cartilage (ec), mild synovial membrane proliferation (mip) and superficial notched cartilage (snc) were detected. Scale bar; 200 μm. (B) Bar graph illustrating histological lesion score for paw joint depending on synovial membrane proliferation, pannus formation and articular cartilage erosion in all studied groups. Bars carrying different superscripts letters (a, b, c, d, e, and f) are significantly different as analyzed by the one-way ANOVA test, followed by the multiple comparisons by Duncan's Post-hoc test ($p < 0.05$); $n = 10$ in each experimental group. Values = mean ± SE.

and a significant ($p < 0.05$) increase in total WBC count compared with the vehicle control group. Oral administration of arthritic rats with BCP at 300 mg/kg for four weeks significantly ($p < 0.05$) increased RBC count, Hb concentration, eosinophils, and monocytes and significantly decreased total leukocyte count compared with the non-treated arthritic group. Intraperitoneal injection of arthritic rats with MTX at 1 mg/kg/week for four weeks significantly ($p < 0.05$) reduced RBC count, Hb concentration, PCV, MCHC, MCH, total WBC count, and lymphocytes and significantly ($p < 0.05$) increased MCV, basophils, eosinophils, and neutrophils compared with the non-treated arthritic group. The co-administration of BCP and MTX for four weeks significantly ($p < 0.05$) increased RBC count, Hb concentration, PCV, MCHC, and leukocyte count and significantly ($p < 0.05$) decreased MCV, basophils, and neutrophils compared with the MTX treated group.

The oral administration of LEF at 10 mg/kg/day for four weeks significantly ($p < 0.05$) decreased RBC count, Hb concentration, PCV, MCH, MCHC, platelet count, total leukocyte count, lymphocytes, and neutrophils and significantly ($p < 0.05$) increased MCV, monocytes, basophils, and eosinophils compared with the arthritic-non treated group. The co-administration of BCP and LEF for four weeks significantly ($p < 0.05$) increased RBC count, Hb concentration, PCV, total leukocyte count, and lymphocytes and significantly ($p < 0.05$) decreased MCV, monocytes, basophils, and eosinophils compared with the LEF-only treated group. The co-treatment of arthritic rats with MTX and LEF for four weeks significantly ($p < 0.05$) decreased RBC count, Hb concentration, PCV, MCHC, platelet count, total leukocyte count, and lymphocytes and significantly ($p < 0.05$) increased MCV, MCH, monocytes, eosinophils, and basophils compared with the control non-treated group. Meanwhile, group IX (MTX + LEF + BCP) showed significant ($p < 0.05$) increase in RBC count, PCV, and total leukocyte count and significant ($p < 0.05$) decrease in MCV, MCH, MCHC, monocytes, eosinophils, and basophils compared with group VIII (MTX + LEF) (Table 2).

3.2.2. Liver function enzymes

The AIA group showed a significant ($p < 0.05$) increase in serum levels of AST, ALT, and ALP compared with the vehicle control group. Monotherapy of the arthritic rats with BCP induced a significant ($p < 0.05$) reduction in serum ALT and ALP levels compared with the non-treated arthritic group. Monotherapy with MTX or LEF as well as their combination induced significant ($p < 0.05$) increase in serum ALT, AST, and ALP levels compared with the AIA group. Meanwhile, the concurrent administration of BCP and MTX and/or LEF significantly ($p < 0.05$) reduced serum ALT, AST, and ALP levels compared with their respective groups (Table 1).

3.2.3. Histopathological findings

3.2.3.1. Spleen. The rats in the vehicle control group exhibited the normal architecture of splenic stroma and parenchyma. After CFA injection in the hind paw, we detected the depleted germinal center of white pulp. The spleen of BCP-treated group had normal white pulp with the germinal center. Meanwhile, monotherapy with MTX or LEF showed marked regression in white pulp as well as a distortion with the expansion of red pulp. The MTX + BCP group showed a mild reduction in white pulp, while the LEF + BCP group showed a moderate reduction in white pulp. The co-treatment of arthritic rats with MTX and LEF for four weeks resulted in severe regression of white pulp and expansion of red pulp with obvious congested sinusoids. In the MTX + LEF + BCP group, we observed only mild regression of white and red pulps with congested sinusoids (Fig. 3A). These findings were confirmed statistically (Fig. 3B).

3.2.3.2. Bone marrow. Bone marrow exhibited normal cellularity of hematopoietic cords in the vehicle control group and BCP group both. Small foci of hematopoietic cells and numerous aggregated fat cells were observed in the AIA group and MTX group. The co-treatment of arthritic rats with MTX and BCP induced moderate hematopoietic cellularity with few aggregated fat cells in the bone marrow. In the LEF group, minimal bone marrow cellularity was observed, including dispersed hematopoietic cells in relation to numerous aggregated fat cells as well as amorphous eosinophilic material and sinusoidal dilatation. Besides, megakaryocytes were hardly distinguished. In the LEF + BCP group, bone marrow displayed foci of hematopoiesis with fewer aggregated fat cells compared with LEF-only treated group. In MTX + LEF group, bone marrow was composed of numerous aggregated fat cells with few scattered hematopoietic cells. The administration of BCP with MTX and LEF showed a reappearance of

Table 2
Effects of oral administration of β-caryophyllene on the hematological parameters of AIA rats treated with methotrexate and/or leflunomide.

Parameters	Groups								
	Vehicle control	AIA	BCP	MTX	MTX + BCP	LEF	LEF + BCP	MTX + LEF	MTX + LEF + BCP
RBC ($\times 10^6$/uL)	7.9 ± 0.06^{a}	7.03 ± 0.12^{c}	7.5 ± 0.21^{b}	5.2 ± 0.23^{f}	6.6 ± 0.21^{d}	4.8 ± 0.06^{g}	5.6 ± 0.03^{e}	3.8 ± 0.05^{h}	4.7 ± 0.12^{g}
Hb (g/dL)	16 ± 0.08^{a}	14.9 ± 0.25^{b}	15.7 ± 0.22^{a}	9.6 ± 0.2^{e}	12.8 ± 0.16^{c}	9.1 ± 0.2^{e}	10.6 ± 0.39^{d}	8.9 ± 0.14^{e}	9.16 ± 0.17^{e}
PCV%	40.6 ± 0.66^{a}	38 ± 0.57^{b}	39 ± 0.57^{ab}	32 ± 0.57^{e}	36 ± 0.57^{c}	31.8 ± 0.44^{e}	34 ± 0.57^{d}	26.2 ± 0.75^{g}	29.5 ± 0.28^{f}
MCV (fL)	51 ± 0.4^{d}	54 ± 0.57 ^{cd}	52 ± 0.57^{cd}	61.6 ± 0.88^{b}	54.6 ± 0.88^{c}	66 ± 0.6^{a}	61.3 ± 1.5^{b}	69 ± 0.18^{a}	62.7 ± 1.8^{b}
MCH (pg)	20.2 ± 0.2^{bc}	21.15 ± 0.59^{bc}	20.9 ± 0.03^{b}	18.4 ± 0.48^{d}	19.4 ± 0.29^{cd}	18.9 ± 0.033^{d}	19.2 ± 0.39c^{de}	23 ± 0.5^{a}	19.5 ± 0.29^{cd}
MCHC (g/dL)	39.5 ± 0.64^{a}	39.2 ± 0.23^{a}	39.8 ± 0.4^{a}	30 ± 1.5^{cd}	35.5 ± 0.5^{b}	28.6 ± 0.33^{d}	30.3 ± 0.3^{cd}	33.6 ± 0.3^{b}	31.3 ± 1.2^{c}
Platel ($\times 10^3$/uL)	613 ± 45^{ab}	677 ± 36^{a}	636 ± 27^{ab}	568 ± 28^{ab}	598 ± 27^{ab}	537 ± 34^{b}	586 ± 29^{ab}	541 ± 40^{b}	574.3 ± 23^{ab}
WBC (10^3/uL)	12.6 ± 0.32^{c}	16.6 ± 0.38^{a}	13.9 ± 0.45^{b}	7.3 ± 0.152^{e}	10.2 ± 0.4^{d}	4.8 ± 0.25^{f}	8.1 ± 0.35^{e}	2.4 ± 0 .1^{g}	4.16 ± 0.23^{f}
Lymphocytes (%)	92.2 ± 0.6^{abc}	95.9 ± 0.1^{a}	94 ± 0.32^{ab}	89 ± 1.7^{cde}	92 ± 0.58^{abc}	85.3 ± 2.4^{ef}	90.4 ± 1.4^{bcd}	82.5 ± 2.17^{f}	86.5 ± 1.04^{def}
Monocytes (%)	3 ± 0.03^{e}	2.1 ± 0.06^{g}	2.6 ± 0.07^{f}	2.2 ± 0.06^{fg}	2.5 ± 0.03^{f}	6.7 ± 0.25^{b}	4.8 ± 0.09^{d}	8.1 ± 0.145^{a}	6 ± 0.17^{c}
Basophiles (%)	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{c}	1 ± 0^{b}	0 ± 0^{c}	1 ± 0^{b}	0 ± 0^{c}	2 ± 0^{a}	1 ± 0^{b}
Eosinophils (%)	2 ± 0.005^{e}	0.2 ± 0.12^{g}	1.3 ± 0.152^{f}	2 ± 0.11^{e}	2 ± 0.32^{e}	6 ± 0.0^{a}	3.4 ± 0.12^{d}	5.3 ± 0.17^{b}	4 ± 0.06^{c}
Neutrophils (%)	2.8 ± 0.26^{c}	1.8 ± 0.03^{ef}	2.1 ± 0.218^{de}	5.8 ± 0.12^{a}	3.5 ± 0^{b}	1 ± 0.06^{g}	1.4 ± 0.03^{fg}	2.1 ± 0.06^{de}	2.5 ± 0.14^{cd}

Values are represented as the mean ± SE ($n = 10$). Vehicle control: subcutaneously injected with 0.25 mL of paraffin oil into the palmar surface of the left hind paw and orally received corn oil; AIA: subcutaneously injected with 0.25 mL of CFA and orally received corn oil; BCP: subcutaneously injected with 0.25 mL of CFA and orally received 300 mg BCP/kg/day in corn oil; MTX: subcutaneously injected with 0.25 mL of CFA and IP injected with 1 mg MTX/kg/weekly; MTX + BCP: subcutaneously injected with 0.25 mL of CFA and co-treated with MTX and BCP; LEF, subcutaneously injected with 0.25 mL of CFA and orally received 10 mg LEF/kg/day; LEF + BCP: subcutaneously injected with 0.25 mL of CFA and co-administered with LEF and BCP; MTX + LEF: subcutaneously injected with 0.25 mL of CFA and co-treated with LEF and MTX and MTX + LEF + BCP: subcutaneously injected with 0.25 mL of CFA and co-administered with LEF, MTX and BCP. All treatments continued for four weeks. Mean values for the same parameter carrying different superscripts (a, b, c, d, e, f, g, h) are significantly different at $p < 0.05$. RBCs: Red blood cells. PCV: Packed cell volume. Hb: Hemoglobin. MCV: Mean corpuscular volume. MCHC: Mean corpuscular hemoglobin concentration. WBCs: White blood cells.

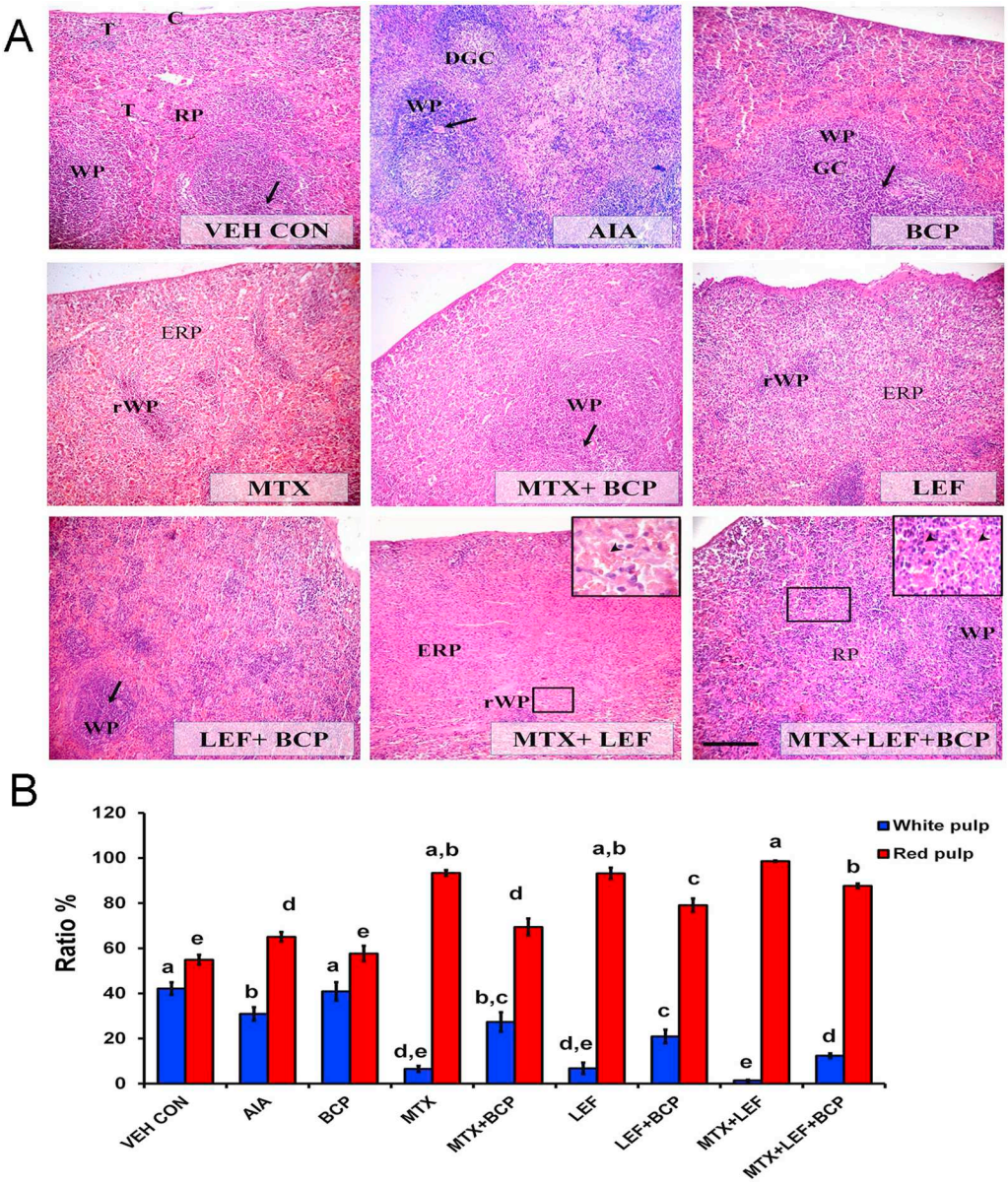

Fig. 3. Evaluation of the splenic changes in rats from different studied groups. (A) Representative photomicrographs from vehicle control (VEH CON), adjuvant-induced arthritis (AIA), β-caryophyllene (BCP), methotrexate (MTX), methotrexate and β-caryophyllene (MTX + BCP), Leflunomide (LEF), leflunomide and β-caryophyllene (LEF + BCP), methotrexate and leflunomide (MTX + LEF), methotrexate, leflunomide and β-caryophyllene (MTX + LEF + BCP) groups showing fibrous capsule (C), fibrous trabeculae (T), white pulp (WP), red pulp (RP), eccentric central arteriole (arrows), germinal centers (GC), depleted germinal centers (DGC), expanded red pulp (ERP), regressed white pulp (rWP) and congested sinusoids (arrowheads). Scale bar; 200 μm. (B) Bar graph illustrating percentage ratios of splenic white and red pulps in all studied groups. Bars carrying different superscripts letters (a, b, c, d, and e) are significantly different as analyzed by the one-way ANOVA test, followed by the multiple comparisons byDuncan's Post-hoc test ($p < 0.05$); $n = 10$ in each experimental group. Values = mean ± SE.

hematopoietic foci with fewer fat cells (Fig. 4A). These results were statistically confirmed (Fig. 4B).

3.2.3.3. Liver. The liver exhibited normal parenchyma in the vehicle control group. The most pronounced changes in the AIA group included the focal necrosed area of hepatocytes infiltrated by numerous mononuclear inflammatory cells. The treatment of arthritic rats with BCP revealed few mononuclear inflammatory cells colocalized with hepatocytes and normal portal area. However, broadened and fibrotic portal area with hyalinized hepatic artery, dilated and congested portal vein, periductal cellular infiltration, severely dilated sinusoid, and many Kupffer cells were detected in the arthritic rats treated with MTX. Portal fibrosis was lower in the MTX + BCP group. We also observed normal hepatocytes and mildly dilated sinusoid with fewer Kupffer cells. In the LEF-treated group, we observed fibrosis of portal area, severely congested sinusoids, and moderate periductal cellular infiltration. However, mild periductal cellular infiltration, dilated portal vein, and fewer Kupffer cells were noticed in the LEF + BCP group. Portal area manifested fibrosis and dilated and congested portal vein. Moreover, disorganized hepatic cord with necrosed hepatocytes was noticed in the MTX + LEF group. Mild fibrotic portal area, moderate periductal cellular infiltration, and numerous Kupffer cells were observed in the MTX + LEF + BCP group (Fig. 5A). These data were statistically significant (Fig. 5B).

4. Discussion

Increased efficacy of RA drugs along with the amelioration of their side effects has great importance in the treatment of the disease. Our experiments revealed that daily oral administration of BCP for 28 days attenuated inflammation in arthritic rats; co-administration of BCP with MTX and/or LEF significantly improved the therapeutic efficacy of MTX and/or LEF and significantly reduced the myelosuppressive and hepatotoxic effects of MTX and/or LEF.

Monotherapy with BCP or MTX or LEF for four weeks significantly reduced the paw thickness and arthritic index and improved histological structures compared with the AIA group. Furthermore, these treatments significantly reduced serum TNF-α level and improved the oxidant/antioxidant status of arthritic rats. The anti-inflammatory effect of BCP was caused by the activation of CB2 receptors [27,28]. BCP has antioxidant activity that reduces reactive oxygen species (ROS) by scavenging hydroxyl anions, lipid peroxides, and superoxide anions

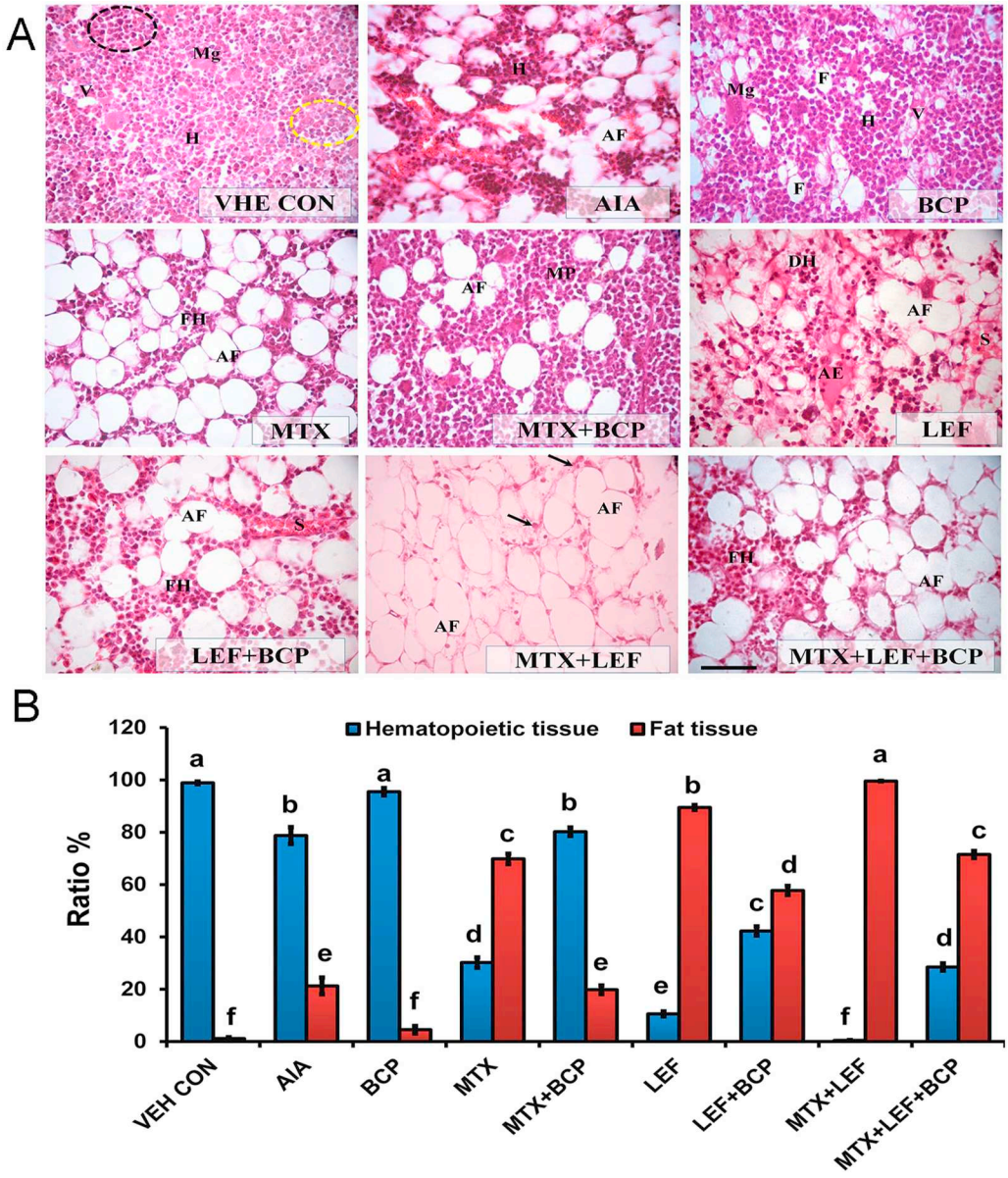

Fig. 4. Evaluation of the rats for changes in bone marrow in the mid-shaft of the femur from different studied groups. (A) Representative photomicrographs from vehicle control (VEH CON), adjuvant-induced arthritis (AIA), β-caryophyllene (BCP), methotrexate (MTX), methotrexate and β-caryophyllene (MTX + BCP), Leflunomide (LEF), leflunomide and β-caryophyllene (LEF + BCP), methotrexate and leflunomide (MTX + LEF), methotrexate, leflunomide an β-caryophyllene (MTX + LEF + BCP) groups showing the normal bone marrow architecture with normal cellularity of hematopoietic tissue (H) and megakaryocytes (Mg), a colony of erythropoietic cells (black circle), a colony of granulopoietic cells (yellow circle), normal vascular sinusoids (V). The individual fat cells (F), aggregated fat cells (AF), sinusoidal dilatation (S), amorphous eosinophilic material (AE), dispersed hematopoietic cells (DH), foci of hematopoietic cells (FH), scattered hematopoietic cells (arrows), the moderate population of hematopoietic cells (MP). Scale bar; 50 μm. (B) Bar graph illustrating the percentage ratio of hematopoietic and fat tissues in all studied groups. Bars carrying different superscripts letters (a, b, c, d, e, and f) are significantly different as analyzed by the one-way ANOVA test, followed by the multiple comparisons by Duncan's Post-hoc test ($p < 0.05$); $n = 10$ in each experimental group. Values = mean ± SE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[29,30]. Similar to our findings, BCP reduced the articular and systemic inflammation in rats with AIA and the oxidative stress in the liver and plasma of the tested animals [4].

The anti-inflammatory effect of MTX may be due to its ability to inhibit the de novo synthesis of purines and pyrimidines [1], which in turn inhibit the production of pro-inflammatory cytokines [32]. T cells isolated from RA patients treated with MTX and activated ex vivo showed a diminished capacity to produce IL-4, IL-3, and TNF-α [31]. MTX scavenges free radicals and attenuates intracellular oxidative stress and consequently mitigates inflammatory response and tissue damage [33]. Our findings are consistent with those of Refaat et al. [34] who found that MTX at 1 mg/kg/week for two weeks significantly regressed inflammation of the hind paw compared with the untreated arthritic rats. A77 1726, the active metabolite of LEF, interferes with the production of inflammatory cytokines by T cells via hindering the activation and expression of nuclear factor-kB [35]. The reduced oxidative stress with LEF treatment in arthritic rats may be due to the suppression of TNF-induced reactive oxygen intermediate generation and lipid peroxidation [36]. Bilasy et al. [9] found that LEF (5 or 10 mg/kg/day) reduced the ankle circumference compared with a control group. Ahmed and Bader [37] stated that arthritic rats treated orally with LEF at 10 mg/kg/day for two weeks significantly reduced serum TNF-α and MDA levels compared with the non-treated arthritic rats. The combination therapy (MTX + LEF) significantly reduced paw thickness and arthritic index compared with the MTX or LEF monotherapy. MTX induces apoptosis of activated T cells, an intervention that would augment the effect of LEF to restrict T cell proliferation [38]. The co-administration of BCP and MTX or LEF or MTX + LEF significantly ($p < 0.05$) reduced paw thickness, arthritic index, and serum TNF-α and MDA levels and significantly increased serum GSH level compared with MTX or LEF or MTX + LEF treatment. These findings indicate the potentiating effect of BCP, MTX and/or LEF.

In our experiment, AIA resulted in a massive increase in total leukocyte count, along with a significant decrease in RBC count, PCV, and Hb concentration. These results are consistent with the findings of Perumal et al. [39]. The treatment of arthritic rats with MTX and/or LEF worsens the hematological status manifested by a significant reduction in RBC count, Hb concentration, PCV, lymphocyte, and total WBC count as well as a significant increase in MCV and eosinophils compared with the arthritic control rats. These changes are in line with the bone marrow findings, in which minimal bone marrow cellularity of the dispersed hematopoietic cells, in relation to numerous aggregated

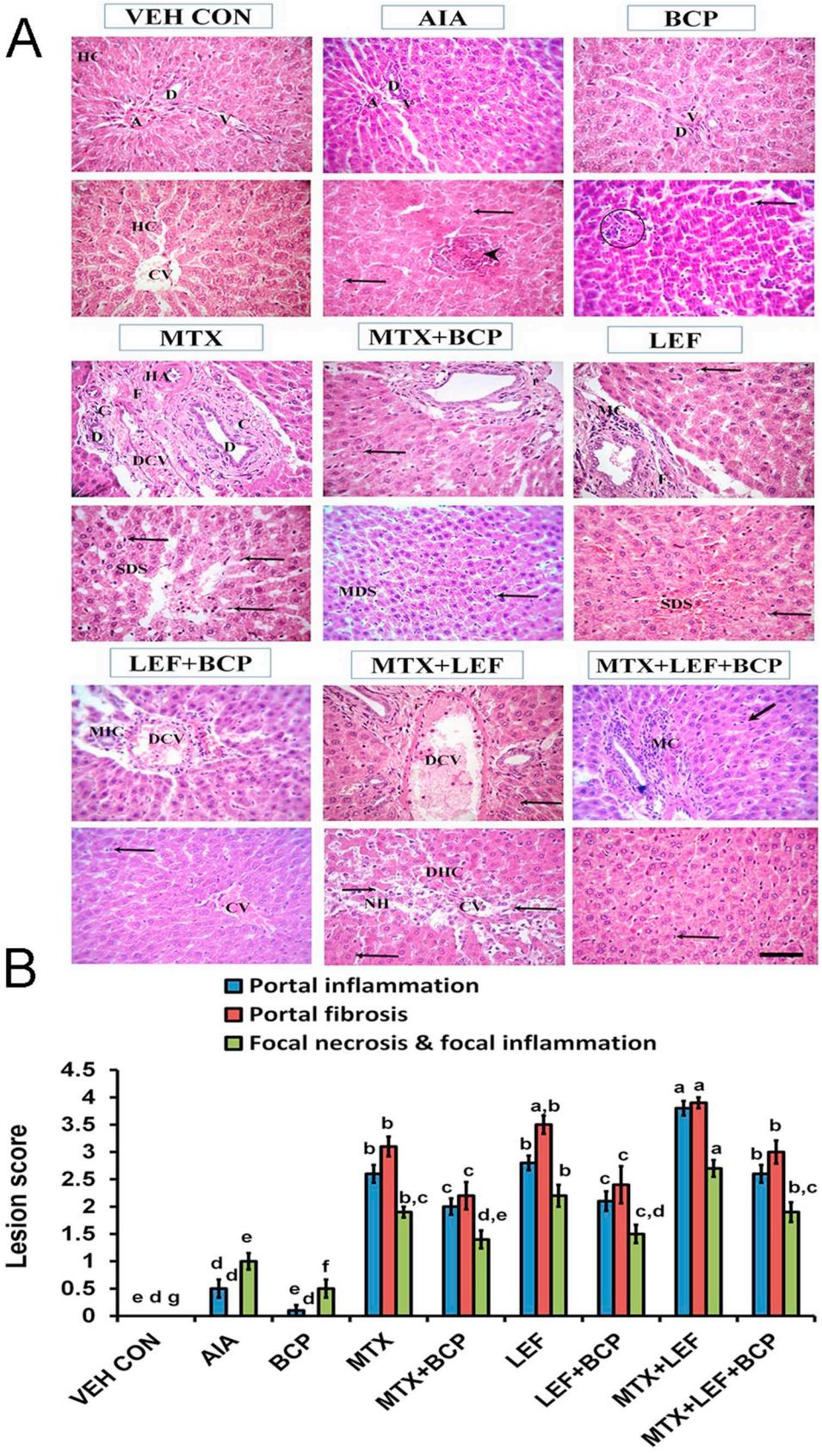

Fig. 5. Evaluation of the changes in the liver of rats from different studied groups. (A) Representative photomicrographs from vehicle control (VEH CON), adjuvant induced arthritis (AIA), β-caryophyllene (BCP), methotrexate (MTX), methotrexate and β-caryophyllene (MTX + BCP), leflunomide (LEF), leflunomide and β-caryophyllene (LEF + BCP), methotrexate and leflunomide (MTX + LEF), methotrexate, leflunomide and β-caryophyllene (MTX + LEF + BCP) groups showing normal hepatic cords (HC) radiated from central vein (CV), normal portal area of hepatic artery (A), portal vein (V) and bile duct (D), focal necrosed area of hepatocytes infiltrated by numerous mononuclear inflammatory cells (arrow head), Kupffer cells (arrows), few numbers of mononuclear inflammatory cells colocalized with hepatocytes (circle), broadened and fibrotic (F) portal area with hyalinized hepatic artery (HA), dilated and congested portal vein (DCV), periductal cellular infiltration (C), severe dilated and congested sinusoids (SDS), reduction in fibrosis of portal area (P), mild dilated sinusoids (MDS), moderate periductal cellular infiltration (MC), mild periductal cellular infiltration (MIC), disorganized hepatic cord (DHC) with necrosed hepatocytes (NH). Scale bar; 50 μm. (B) Bar graph illustrating hepatic lesion score depends on portal inflammation, portal fibrosis, focal necrosis & focal inflammation in all studied groups. Bars carrying different superscripts letters (a, b, c, d, e, f, and g) are significantly different as analyzed by the one-way ANOVA test, followed by the multiple comparisons by Duncan's Post-hoc test ($p < 0.05$); $n = 10$ in each experimental group. Values = mean ± SE.

fat cells as well as amorphous eosinophilic material and sinusoidal dilatation, were detected. These hematological and histopathological changes reflect the myelosuppressive effect of MTX and LEF. The myelosuppressive effect of MTX is due to its effect on the S phase of the cell cycle, which may influence the tissues with high turnover like bone marrow [40]. LEF inhibits the de novo synthesis of pyrimidine in

mitochondria by the enzyme dihydroorotate dehydrogenase and limits the proliferation of activated T lymphocytes [41]. Similar to the aberrant T cell regulation in acquired aplastic anemia, these inconsistent mechanisms imply that a T cell-independent mechanism may be involved in LEF-mediated myelosuppression [42].

The treatment of arthritic rats with BCP at 300 mg/kg for four weeks significantly increased RBC count and Hb concentration and significantly decreased total leukocyte count compared with the non-treated arthritic group. This may be linked to the anti-inflammatory and antioxidant activities of BCP. To some extent, the coadministration of BCP and MTX or LEF or MTX + LEF improved the myelosuppression and bone marrow cellularity compared with MTX or LEF or MTX + LEF treatment. This improvement may be due to the role of the endocannabinoid system in hematopoiesis [43] and the regulation of platelet function [44].

The treatment of arthritic rats with MTX or LEF led to severe functional and structural alterations compared with the non-treated arthritic rats. In comparison to MTX obb0r LEF monotherapy, the combination therapy dramatically increased hepatic toxicity. Our results are consistent with the findings of Bilasy et al. [9]. Another study reported that LEF increased the risk of silent liver fibrosis in patients with RA receiving MTX treatment [45]. The hepatotoxic effect of MTX is due to the metabolite 7-hydroxymethotrexate (7OH MTX), which gets oxidized to a polyglutamate derivative in the liver that induces oxidative stress by depleting NADPH and by interfering with cysteine metabolism [46]. The hepatotoxic effect of LEF and its active metabolite may be due to the inhibition of mitochondrial oxidative phosphorylation complexes and the collapse of mitochondrial membrane potential by modulating adenine nucleotide translocase, which rapidly depletes ATP leading to cell injury [47]. LEF substantially increased MTX and 7OH MTX levels in plasma, lowered their bile elimination, and increased their accumulation in the liver and kidneys [48], exacerbating hepatic toxicity induced by MTX and LEF combination therapy.

Monotherapy of arthritic rats with BCP significantly improved the live structure and function compared with the control rats. This effect may be due to the anti-inflammatory and antioxidant activities of BCP. BCP reduced the protein carbonyl groups and ROS as well as increased GSH levels in the arthritic rats to the levels close to the control [4]. The co-administration of BCP and MTX or LEF or MTX + LEF in the arthritic rats significantly attenuated the hepatotoxic effect compared with the MTX or LEF or MTX + LEF treatment alone.

Taken together, the current study demonstrated that BCP monotherapy can be a potential approach to treat RA. Furthermore, BCP combined with MTX and/or LEF not only potentiates their anti-arthritic effect but also alleviates the extra-articular manifestations in arthritis, which deteriorates with MTX and/or LEF therapy. This study endorses the co-administration of BCP and MTX and/or LEF as an effective therapy for RA.

Declaration of competing interest

The authors declare that they have no conflict of interests.

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