ORIGINAL ARTICLE

Ammodaucus Leucotrichus Seed Extract as a Potential Therapy in Animal Models of Rheumatoid Arthritis Induced by Complete Freund Adjuvant and Chicken Cartilage Collagen

Cheima Djehiche[1] · Nadia Benzidane[1] · Hanene Djeghim[2] · Mehdi Tebboub[3] · Saad Mebrek[2] · Katia Abdelouhab[4] · Abderrahmane Baghiani[1] · Noureddine Charef[1] · Mohammed Messaoudi[5] · Chawki Bensouici[2] · Rabah Lebsir[6] · Talha Bin Emran[7] · Ali Alsalme[8] · David Cornu[9] · Mikhael Bechelany[9,10] · Lekhmici Arrar[1] · Ahmed Barhoum[11]

Abstract

This study assessed the efficacy of an *Ammodaucus leucotrichus* seed extract to treat rheumatoid arthritis in rat models of this disease. Rheumatoid arthritis was induced in rats using two methods: immunization with 100 µL of Complete Freund Adjuvant (CFA) and immunization with 100 µL of a 3 mg/ml solution of type II collagen (CII) from chicken cartilage. The therapeutic potential of the extract was assessed at different doses (150, 300, and 600 mg/kg/day for 21 days in the CII-induced arthritis model and for 14 days in the CFA-induced arthritis model) and compared with methotrexate (MTX; 0.2 mg/kg for the same periods), a commonly used drug for rheumatoid arthritis treatment in humans. In both models (CII-induced arthritis and CFA-induced arthritis), walking distance, step length, intra-step distance and footprint area were improved following treatment with the *A. leucotrichus* seed extract (all concentrations) and MTX compared with untreated animals. Both treatments increased the serum concentration of glutathione and reduced that of complement C3, malondialdehyde and myeloperoxidase. Radiographic data and histological analysis indicated that cartilage destruction was reduced already with the lowest dose of the extract (100 mg/kg/dose) in both models. These results show the substantial antiarthritic potential of the *A. leucotrichus* seed extract, even at the lowest dose, suggesting that it may be a promising alternative therapy for rheumatoid arthritis and joint inflammation. They also emphasize its efficacy at various doses, providing impetus for more research on this extract as a potential therapeutic agent for arthritis.

Extended author information available on the last page of the article
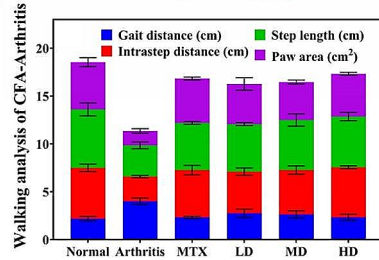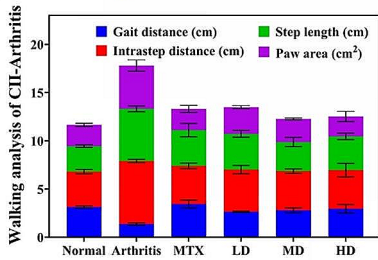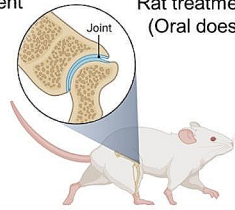
Graphical Abstract

Introduction

Rheumatoid arthritis is a severe autoimmune disease characterized by chronic inflammation that mainly affects the synovial joints in a symmetrical pattern [1]. The presence of autoantibodies, such as anti-citrullinated peptide antibodies, is a distinctive feature of rheumatoid arthritis [2]. In this inflammatory disease, various immune cells, including activated T cells, B cells, and macrophages, release proinflammatory cytokines. These cytokines can stimulate synovial fibroblasts that will cause tissue damage by producing pro-inflammatory cytokines and other factors that induce joint destruction, for instance receptor activator of nuclear factor-kB ligand (RANKL). Immune cells also promote the activity of osteoclasts that degrade bone tissue [3].

The current European Alliance of Associations for Rheumatology (EULAR) guidelines recommend disease-modifying antirheumatic drugs for rheumatoid arthritis management, particularly methotrexate (MTX) as initial therapy [4]. However, MTX has some limitations, such as its poor solubility in water, limited bioavailability, narrow therapeutic window, and suboptimal tissue distribution [5]. MTX also has important adverse effects, including severe neutropenia, hepatotoxicity, and bone marrow suppression [6, 7]. Consequently, there has been a growing interest in exploring alternative treatments for rheumatoid arthritis, by focusing particularly on medicinal plants.

Ammodaucus leucotrichus, known as "hairy cumin" or "kammûn es-sofi" in Algeria, is a fragrant, herbaceous annual plant with a unique smell reminiscent of *Cuminum cyminum* [8]. Recent research highlighted its antioxidant, antibacterial, antifungal, antidiabetic, anti-

inflammatory, anticholinesterase, and cytotoxic properties [9, 10]. Luteolin-7-O-(6"-malonylglucoside) and flavonoid derivatives, such as ammolactone-A and luteolin-A, are among the most commonly detected secondary metabolites in this plant. The fruits have a high lipid content (11 g/100 g dry weight), including fatty acids, such as linoleic acid (C18:2n6), oleic acid (C18:1n9), linolenic acid (C18:3n3), and palmitic acid (C16:0) [8].

In this study, a cold maceration technique was used to extract compounds from *A. leucotrichus* seeds. The anti-arthritis activities of the extracted material and of MTX (for comparison) were then tested in vivo in two frequently used rat models of rheumatoid arthritis: Complete Freund Adjuvant (CFA)-induced and type II collagen (CII)-induced arthritis [11]. Animal models of rheumatoid arthritis are used to understand the disease pathophysiology, investigate the effects and mechanisms of action of candidate treatments, and identify potential therapeutic targets and biomarkers [12]. The positive effect of the extract (at different doses) on joint inflammation/histological alterations, motor functions and inflammatory markers suggest that this *A. leucotrichus* seed extract may represent a groundbreaking therapeutic approach to minimize the adverse effects of the current treatments for rheumatoid arthritis. This study effectively addressed the pressing need of novel and more efficient treatments.

## Materials and Methods

Materials

A. leucotrichus seeds were collected from the arid Algerian Desert, specifically in the Bechar region in the southwest of Algeria, during the summer of 2021. The seed authenticity was verified by Professor Sabah Charmat, Ferhat Abbas University Setif 1, Algeria, and a voucher specimen was officially recorded with the reference number 220/SNV/DA/ UFAS/21. All reagents were of analytical grade and used as received, without any further purification, except for CII that was isolated from chicken cartilage obtained from butchers in the wilaya of Constantine, Algeria. Cartilage was frozen until use. Pepsin from porcine stomach mucosa (EC 3.4.23.1; Spectrum Chemical, USA) was in powdered form and displayed an activity of 750 units per milligram of dry matter. Acetic acid (CH$_3$CO$_2$H, 99.7%), sodium dodecyl sulfate (SDS, 98.5%), N, N,N′,N′-tetramethyl ethylene diamine (TEMED, 99%) and CFA (F5881-10ML) were from Sigma-Aldrich, St. Louis, MO, USA.

Extraction of Phytoconstituents

The collected *A. leucotrichus* seeds underwent a series of preparation steps, including cleaning, air-drying, and grinding with an electric grinder to produce a fine powder. Then, the extract was obtained according to [13]. Briefly, the seed powder underwent methanol extraction at room temperature for 7 days. Then, the solution was filtered, and methanol removed by rotary evaporation at 45 °C. The resulting material was dried at 40 °C to yield the extract (Fig. 1A).

Preparation of Type II Collagen

Chicken sternal cartilage, stored at -20 °C, was thawed and rigorously cleaned. This involved washing with distilled water, followed by immersion in a methanol-chloroform solution (2/1, v/v) for 5 min to eliminate non-collagenous proteins and lipids. CII was prepared following the method by Akram and Zhang [14], with minor adjustments (Fig. 1B). The cartilage pieces were immersed in a mixture of 20% NaCl and 0.05 M Tris-HCl (pH 7.5), 1:10 (w/v) ratio and stirred overnight. This was followed by rinsing with distilled water. The resulting cartilage material was stored at -20 °C until needed. Pepsin-soluble CII was extracted following the procedure by Khong et al. [15], with some modifications. The insoluble material was dissolved in 0.5 M acetic acid (1:100, w/v) and digested with 10% (w/v) pepsin at 4 °C for 48 h. Pepsin-soluble CII was centrifuged at 13,000 g for 1 h. Then, 5 M NaCl was gently added to the supernatant under constant stirring to reach a final concentration of 0.8 M. To neutralize pepsin activity, 2 M NaOH was added to reach a pH=7. The ensuing precipitate was collected and dissolved in 0.1 M acetic acid. After overnight dialysis against distilled water at 4 °C, CII was lyophilized and stored at -20 °C until use. The purity of the purified CII was confirmed by the characteristic UV spectra within the 190–280 nm range, based on the description by Sorushanova, A et al. [16], and by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels [17, 18]. Before use, the CII powder was dissolved in 0.1 M acetic acid, in a SpeedVac vacuum concentrators at 35 °C for 2 h, followed by overnight stirring at 4 °C. This solution was then centrifuged at 3000 rpm for 20 min to eliminate undissolved particles.
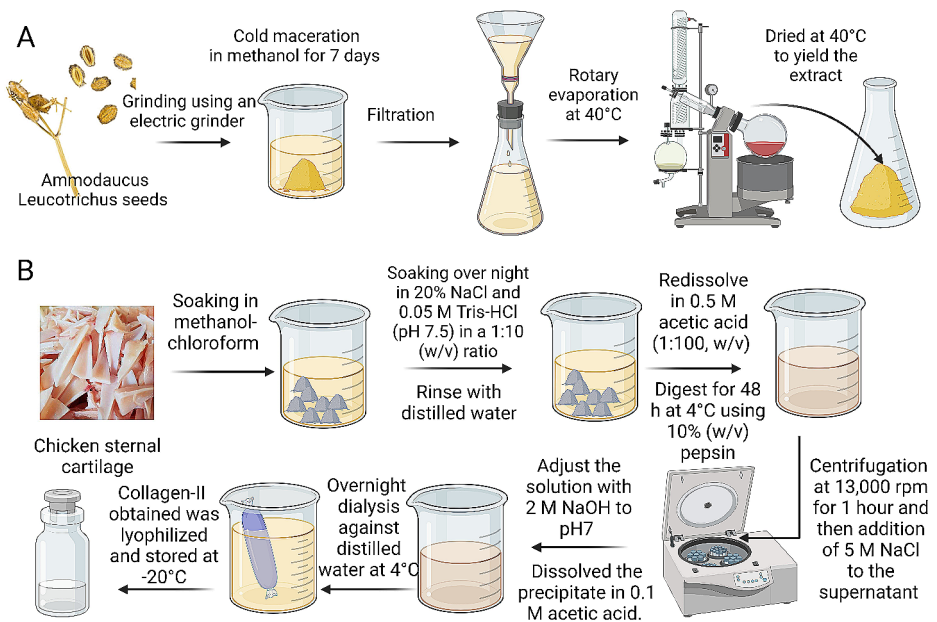
Fig. 1 Schematic figure showing: (A) The extraction of polyphenols from *Ammodaucus leucotrichus* seeds; (B) Type II collagen preparation from chicken cartilage. Overnight soaking; at a 1:10; Digest with 10% (w/v) pepsin at 4 °C for 48 h; Adjustment with 2 M NaOH to pH 7; Precipitate resuspension in 0.1 M acetic acid; Lyophilization of the obtained type II collagen and storage at -20 °C

Animal Tests

Adult male Wistar rats, weighing between 200 and 250 g, were purchased from the Institut Pasteur d'Algérie, Algiers, Algeria. Animals were housed individually in plastic cages at the Biotechnology Research Center (BRC) for 7 days, in standard animal facility conditions (12-hour light and 12-hour dark cycle, temperature of 23 ± 2 °C, and continuous access to food and water) before the experiment start. All animal experimental procedures were performed only after obtaining the necessary clearance by the Institutional Ethics Committee. Animal experiments were carried out in full compliance with the ethical principles laid out by the Algerian Committee for Control and Supervision of Experiments on Animals.

Acute Toxicity Test

Toxicity experiments in male rats were performed following the Organization for Economic Cooperation and Development (OECD) guideline 425 [19]. After the familiarization period, rats were divided in two groups: control group (single dose of 0.9% saline solution by gavage; n)=10 and treated group (single dose of 2000 mg/kg of extract by gavage; $n = 10$). Throughout the study period (14 days), all animals were closely monitored to record any clinical symptoms (e.g. weight changes, death) or behavioural changes [20].

Testing the Extract Effects in the CII-Induced Arthritis Model

Arthritis was induced in male rats following the method described by Lei et al. [21] with minor modifications. First, CII (6 mg/ml) was dissolved in 0.1 M acetic acid overnight and then mixed with an equal volume of CFA to create a viscous emulsion with a final CII concentration of 3 mg/ml. The hind limbs of the rats were shaved and disinfected with 70% (v/v) alcohol. For the initial immunization, 200 µl of the CII-CFA emulsion was injected intradermally at three separate sites in each rat under light diethyl ether anesthesia: 100 µl at the tail base, 50 µl in the left hind leg, and 50 µl in the right hind leg. After 14 days, a booster immunization was carried out by intradermal injection of 100 µl of the CII-CFA emulsion via intradermal injection. Rats were divided in six groups ($n = 7$ rats/group): (i) healthy controls (immunization with 200 µl of 0.1 M acetic acid); (ii) CII-induced arthritis (CII-induced arthritis without any treatment); (iii) CII-MTX (CII-induced arthritis treated with 0.2 mg/kg MTX three times per week by gavage [22] from day 21 to day 42); (iv) CII-HD (CII-induced group arthritis treated with 600 mg/kg/day of the extract); (v) CII-MD (CII-induced group arthritis treated with 300 mg/kg/day of the extract); and (vi) CII-LD (CII-induced group arthritis treated with 150 mg/kg/day of the extract). The *A. leucotrichus* seed extract was orally treated by gavage from day 21 to day 42.

Testing the Extract Effect in the CFA-Induced Arthritis Model

The CFA-induced arthritis model was based on a prior study with minor adjustments [23]. Briefly, male rats received one intradermal injection of 100 µl CFA in the left and right hind legs under mild diethyl ether anesthesia. After 7 days, a booster intradermal injection of 50 µl CFA was administered in both hind legs. Then, rats were divided in six groups ($n = 7$ rats/group): i) healthy controls (immunization with 100 µl of 0.9% NaCl); ii): CFA-induced

arthritis (CFA-induced arthritis without any treatment); iii) CFA-MTX (CFA-induced arthritis treated with 0.2 mg/kg MTX three times per week by gavage [22] from day 14 to day 28); iv) CFA-HD (CFA-induced group arthritis treated with 600 mg/kg/day of the extract); v) CFA-MD (CFA-induced group arthritis treated with 300 mg/kg/day of the extract); and vi) CFA-LD (CII-induced group arthritis treated with 150 mg/kg/day of the extract). The *A. leucotrichus* seed extract was orally treated by gavage from day 14 to day 28.

Studied Arthritis Parameters

Arthritis Index

The arthritis index was assessed following the method by Sun et al. [24]. The scoring system was as follows: 0, no redness or swelling; 1, redness and swelling of the little toe joints; 2, redness and swelling of all paw joints and toes; 3, redness and swelling below the ankle joint; 4, redness and swelling of all joints, including the ankle joint. The arthritis index of each rat was the sum of the scores for all joints.

Gait Evaluation

Disease progression in arthritic rats was monitored using the method developed by Gurram et al. [25]. Changes in the rats' walking pattern were detected by dipping their hind paws in blue ink and then letting them walk on paper that absorbs ink. The footprints on paper were scanned and analyzed to measure step length, intra-step distance, gait distance, and surface area. The ImageJ software (Version 1.44; Wayne Rasband, National Institute of Health) was used for these measurements.

Measurement of Body, Spleen, and Thymus Weight

Rats were weighed weekly throughout the experiment and were euthanized under diethyl ether anesthesia at the study end. Spleen and thymus were removed and weighed.

Biochemical Parameters

After sacrifice, blood samples were collected by cardiac puncture and centrifuged at 800 g for 10 min to obtain serum that was stored at -20 °C until use [26].

Serum Levels of Complement 3 (C3)

C3 levels in serum was determined with an enzyme-linked immunosorbent assay (ELISA) using the Mindray BS-240 Pro sequential analysis system [27].

Reduced Glutathione Level

The concentration of reduced glutathione (GSH) in serum was determined using the method described by Ellman [28]. Compounds with sulfhydryl groups turn yellow when DTNB is added. After adding 3 ml of 4% sulfosalicylic acid and 0.2 ml of supernatant to the tubes,

they were centrifuged at 2500 g for 15 min. Then, 0.2 ml of the supernatant was mixed with 1 ml of phosphate buffer (0.1 M, pH 7.4) and 0.4 ml of 10 mM DTNB. The absorbance at 412 nm was measured.

Serum Levels of Oxidative Stress Markers

Malondialdehyde (MDA) concentration was measured to determine the rate of lipid peroxidation using the approach proposed by Mas-Bargues et al. [29]. After adding 0.5 mL of tricarboxylic acid (20%) and 1 mL of thiobarbituric acid (0.67%) to 0.5 mL of serum, the mixture was incubated at 95 °C for 1 h and then cooled in an ice bath for 10 min. Then, samples were vortexed and centrifuged at 10,000 rpm for 15 min. The supernatant absorbance was measured at 532 nm. Myeloperoxidase (MPO) activity was measured using the O-dianisidine method in H_2O_2. Each sample was combined with 0.167 mg/mL O-dianisidine in 2.9 ml of phosphate buffer (50 mM, pH = 6) at a volume of 0.1 ml. The absorbance for each sample was measured at 470 nm every minute for 3 min.

X-ray Analysis of CII- and CFA-Induced Arthritis Severity

At the treatment end, rats were euthanized, and their hind legs fixed in 10% formalin to assess joint deterioration by X-ray imaging. The modified Sharp/Van der Heijde system [30] was used for scoring radiographic images. Joint space narrowing was scored as follows: 0, no narrowing; 1, minimal narrowing; 2, loss of 50% of joint space; 3, loss of 75% of joint space; and 4, complete loss of joint space. Erosion was scored as follows: 0, no change; 1, discrete but clear presence of erosion; 2 or 3, more extensive erosion, based on the affected surface area of the joint; 4, significant erosion or multiple erosions; 5, complete joint collapse or erosion of the whole joint surface [31].

Histopathological Analysis of Hind Legs

The histopathological analysis of hind legs initiates with the careful retrieval of each rat's paws upon sacrifice. Subsequently, the paws are promptly immersed in 10% formalin solution for a minimum duration of 48 h to ensure adequate fixation. Following fixation, a meticulous decalcification process is conducted using 15% nitric acid over one week to remove mineral deposits. Once decalcification is complete, the paws undergo a precisely controlled series of alcohol and xylene baths within an automated system, culminating in their embedding within paraffin blocks. After embedding, thin histological sections of the joint are meticulously prepared for each paw and affixed onto glass slides. These slides are then subjected to staining with hematoxylin and eosin (H&E) for optimal morphological visualization under an optical microscope. Hematoxylin imparts a blue or violet hue to cellular nuclei, while eosin imparts shades of pink or red to cytoplasmic and extracellular structures. The application of H&E staining facilitates comprehensive examination of cellular and tissue structures within rat paws, enabling detailed histopathological analyses [32].

Statistical Analysis

All results were expressed as mean±standard deviation (SD). Groups were compared with one- and two-way ANOVA, followed by the Tukey's post hoc test for multiple comparisons. The statistical analyses were performed using SPSS Statistics 26.0 (IBM, USA) and figures were generated using GraphPad Prism (version 8.02, GraphPad Software, USA). P values<0.05 were considered significant.

Results

CII Purity

CII purity is essential to ensure the reliability of the animal models used in our study and thus the precise evaluation of the anti-arthritic properties of the tested extract. To assess purity, the structural characteristics of CII obtained from chicken sternum cartilage were investigated by UV absorption spectrum analysis and SDS-PAGE. The UV absorption spectrum of CII displayed two distinct absorption peaks at approximately 225 nm and 280 nm (Fig. 2). The peak at 225 nm is associated with the absorption of aromatic amino acids, primarily tryptophan. The peak at 280 nm corresponds to the absorption of UV-light by the peptide bonds in the protein backbone [33]. This unique peak is due to the relatively low concentration of tyrosine residues in CII, estimated at approximately 8.0-9.7 per 1000 amino acid residues. SDS-PAGE confirmed CII purity by showing the presence of the α1
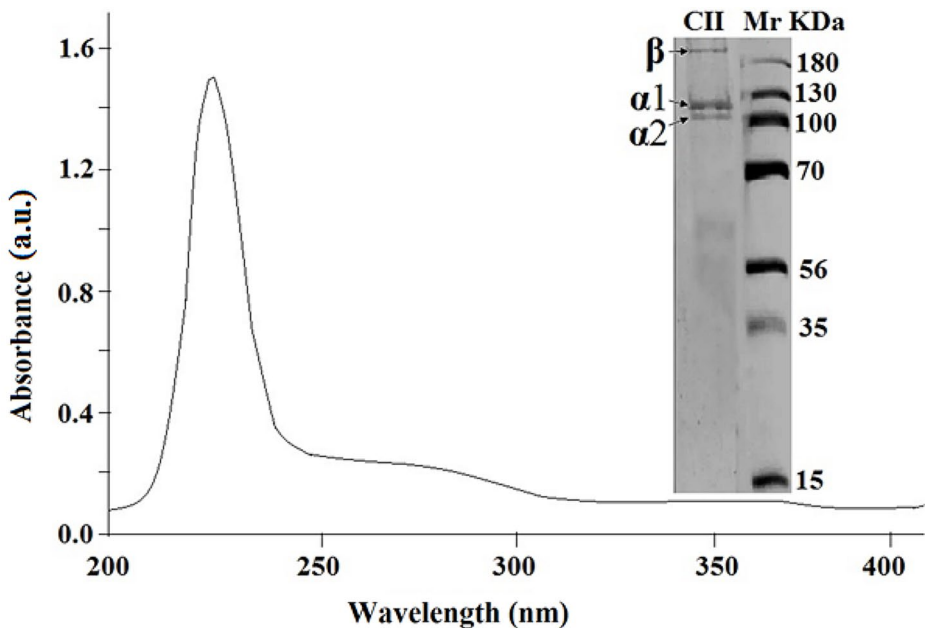
Fig. 2 UV absorption spectrum and SDS-PAGE analysis of type II collagen obtained from chicken sternum cartilage. Mr: Molecular weight markers (15–180 KDa)

and α2 chains, which are hallmark features of CII, and of the β chain. This confirmed that the sample was CII and its high purity.

Extract Composition

A preliminary gas chromatography-mass spectrometry analysis of the *A. leucotrichus* seed extract identified several significant compounds that are listed in Table 1. The most abundant compound was 9-octadecenoic acid ($\sim 30\%$ of the extract composition). This compound, also known as oleic acid, is a monounsaturated fatty acid that has various health benefits, including anti-inflammatory and antioxidant properties. Moreover, n-hexadecanoic acid, hexadecanoic acid, methyl ester, 9(E)-octadecenoate, and (Z, Z)-9,12-octadecadienoic acid were detected. These compounds contribute to the overall chemical profile of the extract and may possess bioactive properties. Their identification suggests that the extract may have various pharmacological effects.

In vivo Acute Toxicity Test

Before proceeding with the in vivo assessment of the *A. leucotrichus* seed extract anti-arthritis effect, an acute toxicity test was carried out to determine the appropriate extract concentrations to be used. Male rats received one dose of the extract (2000 mg/kg/day) by oral gavage and then they were monitored for 14 days. No rat died and no sign of acute toxicity (e.g. excessive salivation, diarrhea, or respiratory problems) was observed. The LD50, which denotes the dose at which 50% of the tested subjects die, was not reached at the tested dose. This indicates that the extract is relatively safe even at high doses [20].

In vivo Anti-Arthritic Effect of the Extract

To assess its potential anti-arthritic effect, three doses of the *A. leucotrichus* seed extract (150, 300, and 600 mg/kg) were used in two well-established rat models of arthritis (CII-induced arthritis and CFA-induced arthritis). These models closely mimic the clinical features of human rheumatoid arthritis [34].

Arthritis Index and Body Weight

Rheumatoid arthritis manifests visible signs of joint inflammation, such as redness, swelling, and edema, which serve as indicators of disease severity [18]. In our experiments, we closely monitored these parameters in rats with CII-induced and CFA-induced arthritis (Fig. 3). Specifically, we assessed paw swelling, changes in body weight, potential altera-
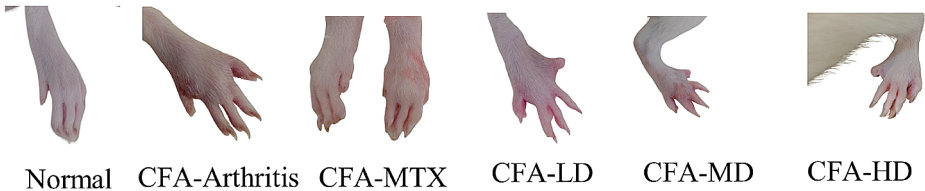
Table 1 Chemical formula, molecular weight, and weight% (g/100 g of extract) of the major compounds in the *Ammodaucus leucotrichus* seed extract	Compounds	Chemical formula	Molecular weight, g	Weight, %
	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	3.86
	Methyl-hexadecanoic acid	$C_{17}H_{34}O_2$	270.5	4.87
	Methyl 9(E)-octadecenoate	$C_{19}H_{36}O_2$	296.5	8.87
	(Z, Z)-9,12-octadecadienoic acid	$C_{18}H_{32}O_2$	280.4	8.77
	9-octadecenoic acid	$C_{18}H_{34}O_2$	282.5	29.69

(A) CII-induced Arthritis (42 days)

Normal   CII-Arthritis   CII-MTX   CII-LD   CII-MD   CII-HD

(B) CFA-induced Arthritis (28 days)

Normal   CFA-Arthritis   CFA-MTX   CFA-LD   CFA-MD   CFA-HD

Fig. 3 Paw swelling in healthy controls (normal) and untreated and treated rats with CII-induced (**A**) and CFA-induced arthritis (**B**) at the end of the experiment (day 42 and day 28, respectively). MTX, methotrexate (0.2 mg/kg); LD, 150 mg/kg of the extract; MD, 300 mg/kg of the extract; HD, 600 mg/kg of the extract

tions in behaviour, and arthritis index. Following arthritis induction (first immunization), the arthritis index progressively increased in both CII-induced and CFA-induced arthritis models, accompanied by paw swelling (Fig. 4A-B and Table S1), compared to healthy controls. The observed weight change in the initial 7 days of the study can be attributed to various factors. Initially, it's essential to recognize that rats, like most organisms, undergo natural growth and adaptation to their environment. The weight change likely reflects a combination of these factors and regular food intake by the rats. Furthermore, weight gain slowed from day 21 to day 42 in CII-induced arthritis rats and from day 14 to day 28 in CFA-induced arthritis rats compared to healthy controls (Fig. 4C-D and Table S1). This decline may be attributed to the onset of joint inflammation induced by immunization with CFA. The inflammatory response triggered by CFA arthritis may cause discomfort and movement difficulties in the rats, subsequently affecting their food intake and resulting in weight loss during this period. Our results confirmed the development of arthritis following injection of CII and CFA. In treated rats (administered with either the A. leucotrichus seed extract or MTX), paw swelling, arthritis index, and body weight were less affected compared to untreated rats (Fig. 4 and Table S1). This suggests that treated rats displayed signs of recovery, notably from day 28 to day 42 in the CII-induced arthritis group and from day 21 to day 28 in the CFA-induced arthritis group. These findings indicate that the A. leucotrichus seed extract and MTX limited arthritis progression and improved the overall condition of the animals.

Gait Changes

At the end of the experiment (day 42 for the CII-induced arthritis model and day 28 for the CII-induced arthritis model), the following walking parameters were measured in healthy rats (normal) and in the different groups of rats with arthritis: gait distance, intra-step dis-
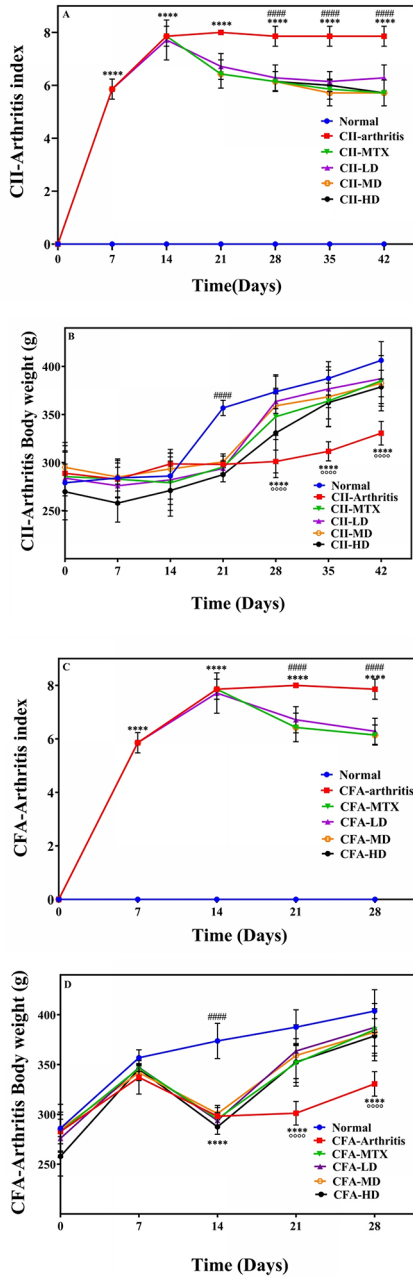
Fig. 4 Effect of the extract (different doses) on the arthritis index (extent of joint redness and swelling) (**A, C**) and body weight (**B, D**) compared with methotrexate (MTX; 0.2 mg/kg). (**A, B**) CII-induced arthritis and (**C, D**) CFA-induced arthritis. Data are the mean±SD (*n*=7 rats/group) (two-way repeated measures ANOVA, followed by Tukey's multiple comparisons test). LD, 150 mg/kg of the extract; MD, 300 mg/kg of the extract; HD, 600 mg/kg of the extract

tance, step length, and paw print area [35] (Fig. 5 and Table S2). Compared with the healthy control groups, the gait distance increased in the CII-induced- arthritis and CFA-induced arthritis groups (2.17 ± 0.23 cm versus 4.54 ± 0.34 cm and 3.99 ± 0.34 cm, respectively). In animals treated with MTX or the extract, gait distance decreased compared with untreated rats. In both CII- and CFA-induced arthritis groups, treatment with the different doses of the extract (LD, MD, and HD) suggested a dose-dependent impact on locomotor activity.
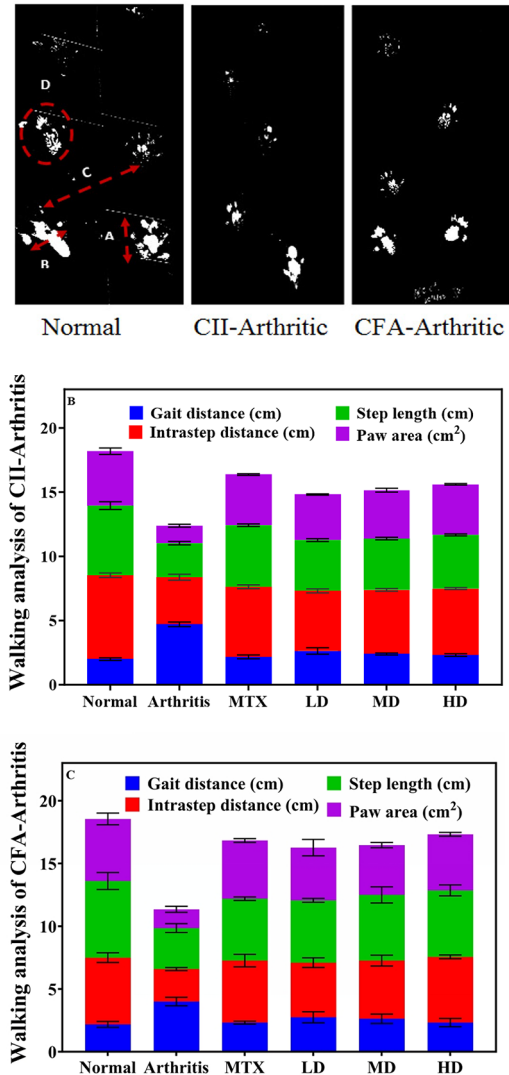
Fig. 5 Walking parameter analysis at the study end. (A) Comparison of hind paw prints in healthy controls (normal) and rats with CII-induced arthritis and CFA-induced arthritis. Effects of the extract on gait distance, intra-step distance, step length, and paw print area of rats with (B) CII-induced and (C) CFA-induced arthritis. Data are the mean$\pm$SD ($n=7$/group). MTX, methotrexate (0.2 mg/kg); LD, 150 mg/kg of the extract; MD, 300 mg/kg of the extract; HD, 600 mg/kg of the extract

Compared with the healthy control group, intra-step distance decreased in the CII-induced-arthritis group and CFA-induced arthritis groups (5.31 ± 0.38 cm versus 3.20 ± 0.36 cm and 2.59 ± 0.11 cm, respectively). In rats treated with MTX or the extract (LD, MD, and HD), intra-step distance increased, and the strongest effect was observed in the HD group for both models (Table S2). Step length (i.e. the distance between successive placements of the same foot) also was reduced in the CII-induced arthritis group (2.86 ± 0.20 cm) and CFA-induced arthritis groups (3.26 ± 0.35 cm) compared with the healthy control group (6.11 ± 0.67 cm). Both treatments improved step length. Paw print area (i.e. the surface area of paw imprints) was reduced in both CII- and CFA-induced arthritis groups compared with the healthy control group (1.87 ± 0.20 cm^2 and 1.49 ± 0.24 cm^2 versus 4.94 ± 0.46 cm^2, respectively). This reduction was limited by treatment with MTX and the extract (all three doses).

These findings suggest that the A. leucotrichus seed extract, especially at higher doses, may have a dose-dependent ameliorative effect on walking parameters, potentially improving locomotor function and motor coordination impairment caused by arthritis. These findings align with previous research [36, 37]. Differences in walking parameters between healthy rats and those with CII-induced and CFA-induced arthritis models are attributed to arthritis-induced pathological changes. Arthritis groups exhibited altered gait parameters compared to healthy controls, with increased gait distance possibly reflecting compensatory mechanisms to alleviate joint discomfort. Conversely, reductions in intra-step distance, step length, and paw print area indicate impaired motor coordination and locomotor function due to arthritis-induced pain and stiffness. Treatment with MTX or the extract, especially at higher doses, mitigated these abnormalities, suggesting therapeutic potential in addressing arthritis-related gait issues. These results support previous studies on the extract's efficacy in mitigating arthritis-related gait abnormalities.

Relative Weight of Spleen and Thymus

The relative weight (% of body weight) of the thymus and spleen were determined in the different groups at the study end to monitor the immune system functionality [38] (Fig. 6 and Table S3). The relative weight of thymus and spleen were increased in both CII- and CFA-untreated arthritis groups compared with healthy controls. The spleen weight increased from 0.188 ± 0.022% in the healthy control group to 0.262 ± 0.004% in the CII-induced arthritis group and to 0.335 ± 0.050 in the CFA-induced arthritis group. Similarly, the thymus weight increased from 0.061 ± 0.005% in the healthy control groups to 0.120 ± 0.002% in the CII-induced arthritis group and to 0.171 ± 0.02 in the CFA-induced arthritis group. These findings are in line with those of previous studies [39, 40]. Both treatments (MTX and the extract at different doses) reduced thymus and spleen relative weights in both models, suggesting a possible restoration of the immune system function, particularly with the extract at the highest dose.

Effect of the Extract on Inflammation Markers

The inflammation markers C3, GSH, MDA, and MPO were measured at the study end (day 42 for CII-induced arthritis and day 28 for CFA-induced arthritis). Serum C3 levels, a marker of inflammation in rheumatic diseases [41, 42], was increased in both CII- and CFA-induced arthritis groups (0.40 ± 0.01 and 0.27 ± 0.01) compared with the normal group
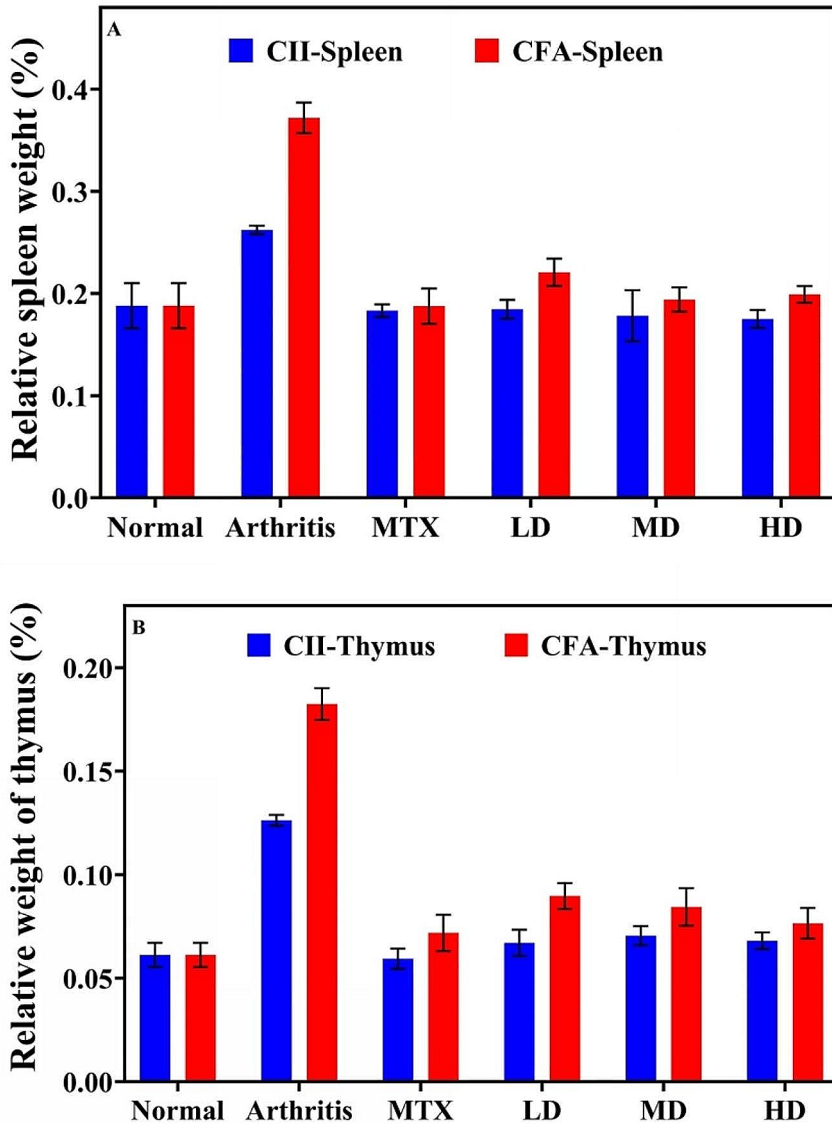
Fig. 6 In vivo therapeutic effect of the *A. leucotrichus* seed extract on (**A**) thymus and (**B**) spleen relative weights in rats with CII-induced (blue) and CFA-induced (red) arthritis. Data are the mean (%)±SD (*n*=7/group); (one-way ANOVA, followed by Tukey's multiple comparisons test). MTX, methotrexate (0.2 mg/kg); LD, 150 mg/kg of extract; MD, 300 mg/kg of extract; HD, 600 mg/kg of extract

(0.20±0.01 g/l) (Fig. 7A and Table S4). In the CII-induced arthritis group, only MTX significantly decreased C3 concentration, but not the extract (all three doses) [42]. Conversely, in the CFA-induced arthritis group, the HD extract reduced C3 concentration to 0.18±0.01 g/l. Conversely, MTX did not have any effect, in line with the findings of Marchi et al. [43] and Troldborg et al. [44].

GSH concentration in serum decreased in the CII- and CFA-untreated groups compared with the normal group (3.36±0.15 μM and 3.56±0.20 μM versus 5.70±0.50 μM, respectively) (Fig. 7B and Table S4). Treatment with the extract and MTX increased GSH concentration almost to normal levels in both models, consistent with previous findings [24, 45, 46]. Specifically, GSH concentrations were 5.00±0.55 μM and 5.03±0.15 μM in the CII-MTX induced and CFA-MTX groups, respectively. In both models, GSH increased progressively with the extract dose: from 4.26±0.75 μM (CII-LD) to 5.33±0.30 μM (CII-HD) and from 4.50±0.40 μM (CFA-LD) to 5.16±0.25 μM (CFA-HD).

The serum concentrations of MDA and MPO, two key inflammation markers for rheumatoid arthritis [47, 48], were increased in the CII-induced arthritis and CFA-induced arthritis groups (Fig. 7C and Table S4): from 0.27±0.01 to Serum MDA and MPO levels considered significant inflammatory markers for rheumatoid arthritis [47, 48], were notably higher in both the CII-induced and CFA-induced arthritic groups compared to the normal group. The concentrations of MDA measured 1.86±0.05 and 1.93±0.06 nM for MDA, and from 11.77±0.20 to 32.85±4.0 and 20±2 ng/ml for MPO, respectively. Treatment with However, the administration of various doses of the extract and MTX demonstrated substantial reduced MDA levels across all treated groups in both models. In the CII-induced arthritis model, HD group exhibited a significant decrease in MPO concentration decreased to 14.12±0.01 ng/ml in the CII-HD,, followed by reductions in the groups treated with CII-MTX, CII-LD, CII-MD, CFA-MD, and CFA-HD, showing concentrations of 16.44±0.16 in the CII-MTX, 17.13±0.11 in the CII-LD, and 16.33±0.15 in the CII-MD group. In the CFA-induced arthritis, MPO concentration decreased to 8.23±0.2 in the CFA-MD group and 8.24±0.25 ng/ml in the CFA-HD group. This indicates that both the extract and MTX contribute to reduce these inflammation markers, suggesting a role in attenuating the oxidative stress and inflammation associated with rheumatoid arthritis.

X-ray Analysis of Joint Space and Erosion

X-ray imaging was used to assess leg swelling, a characteristic sign of inflammation in arthritic conditions, in the different groups at the study end (Fig. 8A and Table S5). The CII- and CFA-induced arthritis exhibited substantial joint erosion (3.33±0.57 in both groups) compared with negative controls (no swelling; 0.00±0.00). Following treatment with MTX or the extract, joint erosion decreased in both models compared with untreated animals: 2.33±0.53 in the CII-MTX group and 2.33±0.56 in the CFA-induced arthritis MTX group and 1.00±0.01 in the CII-HD group and 1.33±0.56 in the CFA-HD group, indicating a reduction in joint erosion due to MTX treatment. The extract-treated groups (LD, MD, HD) in both arthritis conditions showcase a decreasing trend in joint erosion. In the CII-induced arthritis model, the high-dose (HD) group presents the most considerable reduction, measuring 1.00±0.01, while in the CFA-induced arthritis model, the HD group exhibits a measurement of 1.33±0.56. These X-ray results are in line with previous studies [49–51]. The reduction in joint erosion observed in extract-treated groups, particularly in the HD groups,
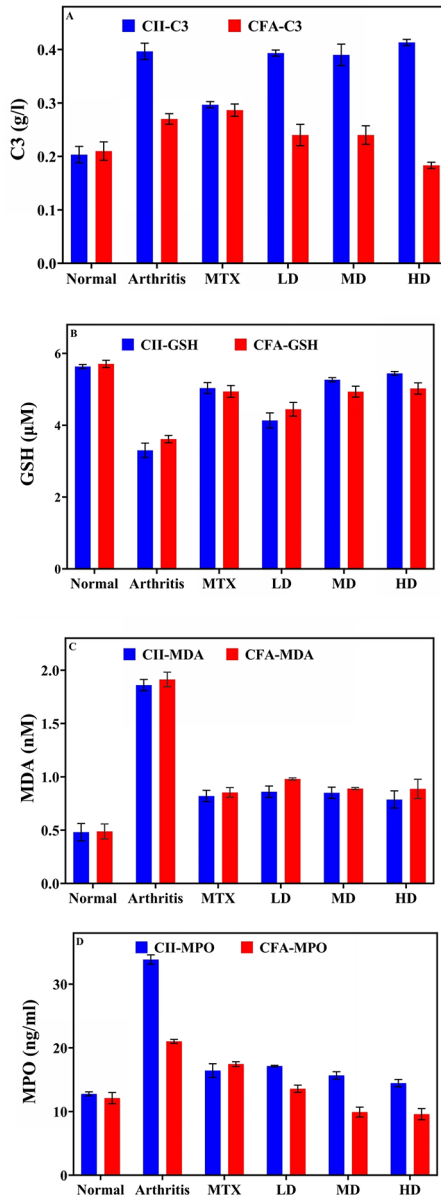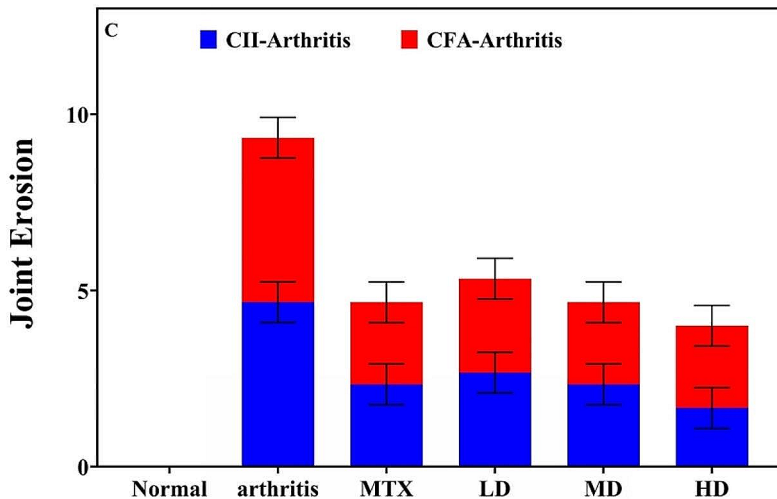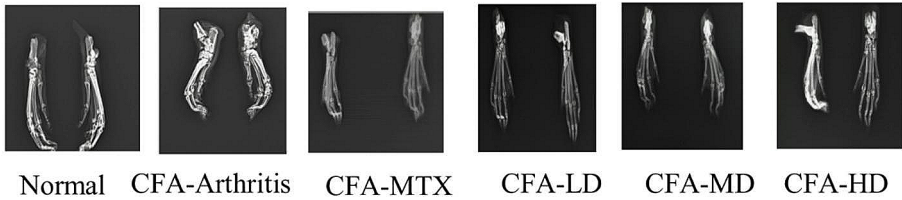
Fig. 7 Effect of the extract on the serum concentration of the following inflammatory biomarkers in CII-induced arthritis and CFA-induced arthritis: (**A**) C3, (**B**) GSH, (**C**) MDA and (**D**) MPO. Values are the mean±SD (n=7 rats/group). MTX, methotrexate (0.2 mg/kg); LD, 150 mg/kg of the extract; MD, 300 mg/kg of the extract; HD, 600 mg/kg of the extract

(A) CII-induced Arthritis (42 days)

| Normal | CII-Arthritis | CII-MTX | CII-LD | CII-MD | CII-HD |

(B) CFA-induced Arthritis (28 days)

| Normal | CFA-Arthritis | CFA-MTX | CFA-LD | CFA-MD | CFA-HD |

Fig. 8 X-ray images of the hind paws in rats with (**A**) CII-induced arthritis, and (**B**) CFA-induced arthritis at the study end. Explain what these images show. (**C**) Joint erosion (mean±SD; $n=7$ rats/group). MTX, methotrexate (0.2 mg/kg); LD, 150 mg/kg of the extract; MD, 300 mg/kg of the extract; HD, 600 mg/kg of the extract

suggests a potential anti-inflammatory effect of the extract that may alleviate swelling associated with arthritic conditions.

Effect of the Extract on Joint Histology

Figure 9 shows histopathological changes in rats with collagen type II (CII)-induced arthritis and complete Freund's adjuvant (CFA)-induced arthritis, along with synovial inflamma-
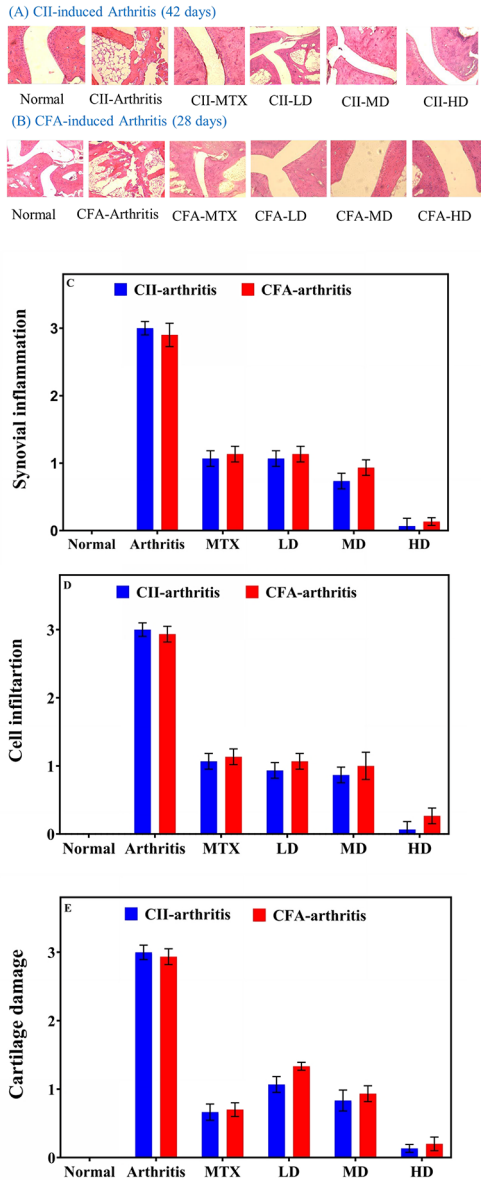
Fig. 9 Histopathological changes in rats with (**A**) collagen type II (CII)-induced arthritis at a magnification of X10 and (**B**) complete Freund's adjuvant (CFA)-induced arthritis at the study's conclusion, also at a magnification of X10. Quantification of (**C**) Synovial inflammation, (**D**) Cell infiltration, and (**E**) Cartilage damage in the different groups. MTX, methotrexate (0.2 mg/kg); LD, 150 mg/kg of extract; MD, 300 mg/kg of extract; HD, 600 mg/kg of extract

tion, cell infiltration, and cartilage damage quantification across treatment groups including methotrexate (MTX) and different doses of extract. Histological analysis of hind leg joint tissue samples after hematoxylin-eosin staining did not highlight any change in the healthy control groups (score = 0 for synovial inflammation, cell infiltration, and cartilage damage). Joints of CII- and Arthritis and CFA-Arthritis) exhibited significant histopathological alterations (score = 3 for synovial inflammation, cell infiltration, and cartilage damage). In the MTX-treated groups, the scores for synovial inflammation (CII-MTX: 1.17 ± 0.03, CFA-MTX: 1.20 ± 0.06), cell infiltration (CII-MTX: 1.13 ± 0.04, CFA-MTX: 1.06 ± 0.01), and cartilage damage (CII-MTX: 0.73 ± 0.02, CFA-MTX: 0.76 ± 0.03) were reduced, indicating a considerable improvement compared with the arthritis groups. In the extract-treated groups, score reduction was dose-dependent. Particularly, in the HD groups the scores for synovial inflammation (CII-HD: 0.10 ± 0.05, CFA-HD: 0.16 ± 0.05), cell infiltration (CII-HD: 0.16 ± 0.02, CFA-HD: 0.40 ± 0.03), and cartilage damage (CII-HD: 0.16 ± 0.03, CFA-HD: 0.23 ± 0.02) were significantly decreased in both models. These results are consistent with the current literature [52–54]. This emphasizes the potential efficacy of the extract in mitigating joint inflammation and damage, particularly at higher doses, similar to the effects observed with MTX.

Conclusion

In this study, the potential of an *A. leucotrichus* seed extract as an antiarthritic remedy was thoroughly examined in two robust animal models of rheumatoid arthritis (immunization with CII and CFA). The effect of the *A. leucotrichus* seed extract at three different doses (150, 300, and 600 mg/kg/day) was compared to that of MTX, one of the initial treatments for rheumatoid arthritis in humans. The primary goal was to determine whether this natural extract is a candidate treatment option for rheumatoid arthritis, a challenging autoimmune condition with limited therapeutic options. The extract (all doses) substantially reduced leg swelling in both models, even more than MTX. Similarly, in all extract-treated groups, the arthritis index decreased, and weight gain tended to be better compared with the MTX-treated groups. The extract also improved the serum concentration of inflammation biomarkers (C3, GSH, MDA, and MPO), particularly at higher doses, in both CII and CFA-induced arthritis models. A dose-dependent decrease in joint histopathology scores, specifically in synovial inflammation, cell infiltration, and cartilage damage, was observed in the groups treated with the extract. Gait analysis revealed improved walking patterns in the extract-treated groups compared with untreated animals. Particularly, the HD dose in both CII- and CFA-induced arthritis models led to gait metric changes that were almost similar to those observed in the MTX-treated groups. Overall, this study underlines the considerable potential of the *A. leucotrichus* seed extract, particularly at the highest tested dose (600 mg/kg), for mitigating arthritis-induced symptoms in rats: leg swelling, body weight, inflammation markers, joint histology, and walking patterns. These findings emphasize the importance of further exploring this extract as a prospective therapeutic agent for rheumatoid arthritis.

Data Availability All data in this manuscript are available upon request.

Declarations

Ethical Approval The research protocol methodology involving experimental animals adhered to ethical guidelines outlined in the Declaration of Helsinki and The Council for International Organizations of Medical Sciences (CIOMS). Approval for these protocols was granted in alignment with ethical health research standards, as stipulated by the Algerian Executive Directive (No 10–90 JORA, dated 18 March 2004), and further complies with the provisions of Law No. 88−08 issued on 26 January 1988, addressing veterinary medicine activities and the safeguarding of animal health (No JORA: 004 of 27-01-1988).

Consent to Participate Participants in this research study provided informed consent by established ethical standards.

Consent to Publish Participants in this research study provided explicit consent for the publication of study findings.

Competing Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors and Affiliations

Cheima Djehiche[1] · Nadia Benzidane[1] · Hanene Djeghim[2] · Mehdi Tebboub[3] · Saad Mebrek[2] · Katia Abdelouhab[4] · Abderrahmane Baghiani[1] · Noureddine Charef[1] · Mohammed Messaoudi[5] · Chawki Bensouici[2] · Rabah Lebsir[6] · Talha Bin Emran[7] · Ali Alsalme[8] · David Cornu[9] · Mikhael Bechelany[9,10] · Lekhmici Arrar[1] · Ahmed Barhoum[11]

✉ Ahmed Barhoum
ahmed.barhoum@science.helwan.edu.eg

1   Laboratory of Applied Biochemistry, Department of Biochemistry, Faculty of Nature and Life Sciences, Ferhat Abbas University of Setif 1, Setif 19000, Algeria

2   Biochemistry Laboratory, Division of Biotechnology and Health, Biotechnology Research Center (CRBt), Constantine 25000, Algeria

3   Department of Mechanical Engineering, Faculty of Science of Technology, University Mentouri, Brothers Constantine 1, Constantine, Algeria

4   Laboratory of Applied Biochemistry, Faculty of Nature and Life Sciences, University Abderrahmane Mira, Bejaia 06000, Algeria

5   Nuclear Research Centre of Birine, P.O. Box 180, Ain Oussera, Djelfa 17200, Algeria

6   Department of Informatique, Faculté de Mathématiques et d'Informatique, Université de Guelma, Guelma, Algeria

7   Department of Pharmacy, Faculty of Allied Health Sciences, Daffodil International University, Dhaka 1207, Bangladesh

8   Department of Chemistry, College of Science, King Saud University, Riyadh, Riyadh 11451, Saudi Arabia

9   Institut Européen des Membranes (IEM), UMR 5635, Univ. Montpellier, ENSCM, CNRS, Place Eugène Bataillon, Montpellier 34095, France

10 Gulf University for Science and Technology, GUST, Mubarak Al-Abdullah, P.O. Box 7207, Hawally 32093, Kuwait

11 Chemistry Department, Faculty of Science, NanoStruc Research Group, Helwan University, Cairo 11795, Egypt