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Anti-arthritic effect of *Distemonanthus benthamianus* extracts against rheumatoid arthritis in rats

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ABSTRACT

Objective: To evaluate the anti-arthritic effect of aqueous and methanolic extracts of *Distemonanthus benthamianus*.

Methods: Monoarthritis was induced by an injection of 0.3 mL zymosan A (0.9% NaCl, *v/v*) in the right posterior knee joints of rats. Then, joint diameter and pain threshold were determined. Polyarthritis was induced by an intracaudal injection of complete Freund's adjuvant and rats were treated from day 14 post 1st complete Freund's adjuvant injection until 28 day. The clinical, hematological, biochemical and oxidative stress parameters were evaluated. In addition, histological analysis of the knee joint was perfomed in both tests.

Results: The aqueous and methanolic extracts of *Distemonanthus benthamianus* at a dose of 500 mg/kg ameliorated zymosan A-induced monoarthritis, as evidenced by reduced joint diameter, increased pain threshold, as well as improved joint architecture. In addition, both extracts of *Distemonanthus benthamianus* markedly increased body weight and pain threshold, while reducing paw edema in polyarthritic rats. They also led to a marked decrease in platelets and white blood cells ($P<0.05$), as well as a significant increase in red blood cells, hemoglobin and hematocrit ($P<0.05$). The aqueous and methanolic extracts of *Distemonanthus benthamianus* significantly reduced alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase activities, while increasing serum protein levels ($P<0.05$) with no significant variation in creatinine level. Moreover, both extracts increased catalase and glutathione activities ($P<0.05$), and inhibited malondialdehyde and nitric oxide production ($P<0.01$ and $P<0.001$) in the liver and kidneys. Histological analysis of the joints showed that both extracts triggered tissue reparation.

Conclusions: *Distemonanthus benthamianus* could be used as a potential candidate in the treatment of rheumatoid arthritis.

KEYWORDS: *Distemonanthus benthamianus*; Rheumatoid arthritis; Anti-arthritis; Complete Freund's adjuvant; Hyperalgesia; Inflammation; Oxidative stress

Significance

The pathogenesis of rheumatoid arthritis remains poorly understood. Additionally, harmful side effects of existing treatments and the increasing incidence of the disease in patients require the discovery of new drugs. The previous works have presented the pharmacological potential of *Distemonanthus benthamianus* against inflammatory diseases. This study shows the anti-arthritic properties of this plant against arthritis pathotypes and could be used as a potential candidate in the treatment of rheumatoid arthritis.

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1. Introduction

Rheumatoid arthritis (RA), mono, and/or polyarthritis depending on the number of joints affected, is an inflammatory autoimmune disease in which the immune system attacks and destroys the joints. Joint destruction is caused by an inflammation of the synovial membrane which leads to articular cartilage and bone erosion. As a result, intensive pain leads to a loss of joint movement which becomes almost immobile[1]. This disabling disease which makes patient movement very difficult can also affect several organs of our body such as the lungs, heart, eyes, and vessels[2]. In addition, its mortality rate is increasing because RA increases the risk of heart attacks and/or strokes[2].

Its pathophysiology, which is mainly characterized by multiple inflammatory processes involves several stimulation pathways. These processes are initiated and maintained following an overproduction of reactive oxygen species (ROS)[3]. Thus, the regulation of ROS production plays an important role in the control of inflammatory diseases. Several enzymes like catalase, superoxide dismutase, and peroxidase, as well as antioxidants [glutathione (GSH)], are used naturally by the body to fight against the overproduction of these ROS which can kill or damage cells. Moreover, many cellular processes namely inflammation, apoptosis, and even proliferation involve several mediators [prostaglandin E_2 (PGE_2), interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α)] after significant stimulation of nuclear factor-kappa B (NF-κB) by ROS[4]. Thus, the initiation, development, and maintenance of the process of RA (mono and/or polyarthritis) are closely associated with the overproduction of ROS and many inflammatory mediators. Due to a poorly understood pathogenesis, a lack of appropriate treatment, the harmful side effects of existing treatments, and the worldwide increase in its incidence, RA is emerging as a public health threat today. Therefore, the discovery of new drugs remains a constant challenge for the scientific world.

Distemonanthus benthamianus (*D. benthamianus*) Baillon, belonging to Caesalpiniaceae family, is known as "Eyen" in the southern region of Cameroon. It is a deciduous tree up to 40 m high and whose trunk can reach 1.20 m in diameter. In Cameroon, it is found in the Moungo subdivision (Littoral region), in the South and East regions of the country. The tree is found in semi-deciduous secondary forests in the West African tropics. It grows on swampy and fallow land as well as on cocoa farms[5]. *D. benthamianus* is traditionally used for the treatment of bacterial, fungal, and viral infections[6] and joint pain[7]. The roots are chewed for oral hygiene in Nigeria[8], while the bark of the plant is used to heal intestinal, nervous, and hematological disorders, and to treat dermatitis and urogenital infections[9]. The bark powder is used against skin conditions. Phytochemical screening of *D. benthamianus* bark extract revealed the presence of secondary metabolites (flavonoids, alkaloids, polyphenols, sterols, saponins, triterpenes, tannins, and phenolic compounds)[10]. King *et al*.[11,12] have isolated flavonols, precisely of type oxyayanine A 2 [2-(2,5-dihydroxy-4-methoxyphenyl)-5-hydroxy-3,7-dimethoxy-4H-1-benzopyran-4-one], oxyayanin B [5,6-dihydroxy-2-(3-hydroxy-4-methoxyphenyl-3,7-dimethoxy-4H-1-benzopyran-4-one)] and ayanine (3,7,4'-trimethylquercetin). Further, isolations of two other flavonols[13], a hexa-substituted flavonol[14], a tetrasubstituted B-ring flavonol[15], and a 3-oxygenated flavonolignoid[16] were also carried out. Our previous work presented the effects of *D. benthamianus* bark on acetic acid, indomethacin, and ethanol-induced ulcers[17], as well as on enteropathogenic *Escherichia coli* 31-induced diarrhea in rats[10]. It has been also shown that the compounds present in the aqueous and methanolic extracts of this plant considerably reduce the serum levels of IL-1β and TNF-α[10] and can block muscarinic receptors and calcium channels[18]. HPLC chromatograms showed the presence of gallic acid in both extracts, while rutin was detected only in the aqueous extract[18]. Although *D. benthamianus* is widely used in traditional medicine and studied by researchers, no scientific study has been reported on the anti-arthritic potential of the plant barks. Therefore, the present study aimed to evaluate the preventive and curative potential of aqueous and methanolic extracts of the barks of *D. benthamianus* in models of monoarthritis and polyarthritis induced respectively by zymosan A and complete Freund's adjuvant (CFA) in rats.

2. Materials and methods

2.1. Collection and extraction of the plant

D. benthamianus (leaves, bark, flowers, and fruit) was collected in Souza (Littoral region in Cameroon) in April, and then identified by Tsabang Nole (a botanist) in comparison with a specimen n° 45488 HCN from the National Herbarium of Yaoundé, Cameroon. The rinds were cut into small pieces, shade dried, and powdered, and the dried powder was kept in an airtight container until extraction. Subsequently, 1 000 g of powder was split into two batches of 500 g each, then each batch was mixed separately with 5 L of methanol (99.9%) and 5 L of distilled water, respectively. The two solutions which were macerated frequently were left at room temperature (20-25 ℃) for 48 h and filtered using cotton fibers and Whatman no. 3 filter paper. The filtrate with methanol was concentrated under reduced pressure (170-180 mbar) using a rotary evaporator at 65 ℃. The aqueous filtrate was left to dry in an oven set at a maximum of 40 ℃. These processes made it possible to obtain 30 g of methanolic extract and 25 g of aqueous extract for respective yields of 6% and 5%[10].

2.2. Animals

Wistar rats were reared under natural ambient conditions at the animal facility of the Department of Animal Biology, Faculty of Science, University of Dschang, Cameroon. They were given a

standard rodent diet, with free access to water. These rats were approximately 3.5 months of age with 150-200 g body weight (b.w.).

2.3. Chemicals

Zymosan A, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), ethylenediaminetetraacetic acid (EDTA), and CFA were purchased from Sigma Chemical Co. (St. Louis, MO, USA), while diclofenac (Olfen-100 SR) and NaCl were bought from a local certified pharmacy. Chemicals and extracts were each mixed in DMSO (5%) and PBS before usage.

2.4. In vivo study

2.4.1. Distribution and treatment of animals

To evaluate the effects of extracts on zymosan A-induced monoarthritis and CFA-induced polyarthritis, 35 animals were distributed into 7 groups of 5 animals each. Group 1 served as normal control; group 2 was administered (10 mL/kg) with a solution of DMSO 5% + PBS (negative control); group 3 received 5 mg/kg diclofenac sodium (positive control); groups 4 and 5 were treated with the aqueous extract of *D. benthamianus* (250 and 500 mg/kg); groups 6 and 7 were treated with the methanolic extract of *D. benthamianus* (250 and 500 mg/kg)[19]. Treatments were administered *per os* before zymosan A or 0.9% NaCl injection.

2.4.2. Zymosan A–induced monoarthritis

According to the protocol described by Mortada and Hussain[20], rats (groups 2 to 7) were first anesthetized by intraperitoneal injection (0.1 mL/100 g b.w.) of thiopental (50 mg/kg), followed by an injection of 0.3 mL of freshly prepared zymosan A (in 0.9% NaCl, *v/v*) into the right posterior knee joint[21]. Animals in group 1 received 0.3 mL of 0.9% NaCl under the same conditions. After zymosan A injection, the animals were observed daily until day 5.

2.4.2.1. Measurement of joint diameter and pain threshold

A digital caliper (Mitutoyo, Japan) was used for the measurement of the joint diameter of each animal 1, 2, 3, 4, 5, 6, 24, 48, 72, 96, and 120 h after zymosan A injection[22], and the algesimeter (Randall Selitto, UGO Basile, Italy) was used to measure pain threshold after mechanical stimulations[23] within the same periods.

2.4.2.2. Histological analysis of the knee joints

On the fifth day, all animals were once more individually anesthetized as previously stated. The injected knee joints were surgically incised and stored in formalin (10%) and PBS. Histological analysis using hematoxylin/eosin staining was performed. Sections were observed (×100 magnification) using a Leica DM500 microscope equipped with a Canon powershot SX620 digital camera for image acquisition. The general protocol for histological study was followed[24].

2.4.3. CFA–induced polyarthritis

Animals (acclimatized for 3 d) were anesthetized as previously described, then 100 μL of CFA was injected into the caudal vein on the first day. A second injection of 50 μL CFA was performed on the second day at the same time, except for group 1 which received a double injection of 0.9% NaCl under the same conditions. All treatments were administered orally from the 14th day post CFA first injection, then they continued daily until the 28th day[25].

2.4.3.1. Measurement of joint diameter, mechanical hyperalgesia, body weight, and arthritis score

The left paw joint diameter was measured in each animal under the same conditions, as previously described[25] on day 0 (baseline) before the CFA injection as well as on days 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27 after the first injection of CFA.

Hyperalgesia was measured with an algesimeter (UGO Basile, Italy) on day 0 before the CFA injection and days 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26 after the first CFA injection.

Arthritis score was assessed based on the number of infected legs, leg inflammation, leg deformity, and the animal's inability to use the legs on days 0, 4, 8, 12, 16, 20, 24, and 28. In addition, the body weight was measured daily using a weighing scale.

2.4.3.2. Organ weight, hematological parameters, biochemical parameters, and oxidative stress

At the end of treatment, each rat was anesthetized with thiopental injection (50 mg/kg, *i.p.*) and sacrificed. Blood was collected into two tubes by catheterization of the abdominal artery. The first tube containing EDTA as an anticoagulant was used to evaluate the hematological parameters (red and white blood cells, hematocrit, hemoglobin, platelets). The second tube which did not contain an anticoagulant was centrifuged (2 500 rpm, 15 min) and serum sampling was used for the evaluation of the biochemical parameters [alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, creatinine, alkaline phosphatase (ALP)][26]. Further, some organs (liver, kidneys, spleen, lungs, and thymus) were removed, cleaned in 0.9% NaCl, dehydrated (absorbent paper) and weighed. The liver and kidney were crushed (PBS), centrifuged (2 500 rpm, 15 min) and the supernatant was taken for the assay of oxidative stress parameters like nitric oxide (NO), malondialdehyde (MDA), catalase, and GSH[27].

2.4.3.3. Histological analysis

The left leg joint was gently removed, free of all fat, and fixed with buffered formalin (10%) mixed with PBS for histological analysis. This analysis was performed according to the standard histological protocols, using hematoxylin/eosin staining and the same equipment (×100 magnification) was used as described above.

2.5. Statistical analysis

The results were analyzed by one-way or two way ANOVA

followed by Tukey *post-hoc* test and/or Bonferroni *post-hoc* test using GraphPad Prism, version 5.03. Data are expressed as mean±standard error of the mean (SEM) and $P<0.05$ was considered significant.

2.6. Ethical statement

Experimental protocols were approved by the Laboratory Committee (Laboratory of Animal Physiology and Phytopharmacology, Department of Animal Biology, Faculty of Science, University of Dschang Cameroon, Protocol No1209004, 16 July 2019) following the standard ethical guidelines for the use and care of laboratory animals (guidelines of the European Community; EEC Directive 86/609/EEC, November 24, 1986).

3. Results

3.1. Effect of D. benthamianus on zymosan A-induced monoarthritis

3.1.1. Effects of D. benthamianus on joint diameter and pain threshold

A marked increase in joint diameter, and a decrease in pain threshold, was observed 1 h after zymosan A injection compared to the normal control group ($P<0.001$). Treatment with both extracts (250 and 500 mg/kg) and with diclofenac (5 mg/kg) decreased the joint diameter compared to the negative control group ($P<0.05$) (Figure 1A). Moreover, an increase in pain threshold in all groups treated with both extracts (250 and 500 mg/kg) and diclofenac (5 mg/kg) was observed from the 1st to the 6th hour and then from the 2nd to the 5th day compared to the negative control group (Figure 1B).

3.1.2. Effects on histological structures of joints

Figure 2 presents the histological sections of joints. Rats in the negative control group showed joints with inflammation of the synovial membrane and cartilage destruction with bone erosion and leukocyte infiltration in comparison to the normal control group (Figure 2B). In contrast, unaltered synovial membrane and well-developed cartilage with proliferative chondrocytes and no leukocyte infiltration were found in the joints of the diclofenac-treated group (Figure 2C), and the groups treated with the aqueous extract (500 mg/kg; Figure 2E) or the methanolic extract of *D. benthamianus* (500 mg/kg; Figure 2G).

3.2. Effect of D. benthamianus on CFA-induced polyarthritis

3.2.1. Effects on joint diameter, pain threshold, and arthritis score

Figure 3A shows the effect of *D. benthamianus* extracts on joint diameter changes after CFA injection. The results show a gradual increase in joint diameter ($P<0.001$) from day 9 compared to the normal control group. The aqueous extract (500 mg/kg) markedly decreased joint diameter compared to the negative control group ($P<0.001$). The maximum effect was obtained on day 25. The methanolic extract at the same dose provoked a significant decrease ($P<0.001$) in joint diameter with the maximum effect obtained on day 27.

Figure 3B shows the effect of aqueous and methanolic extracts of *D. benthamianus* on animals' pain threshold after CFA injection. There was a progressive and significant decrease ($P<0.001$) in pain threshold from day 8 in comparison with the normal control group. The aqueous extract (500 mg/kg) prominently increased the pain threshold compared to the negative control group with the maximum effect obtained on day 26 ($P<0.001$). The methanolic extract at the same dose significantly increased the pain threshold with the maximum effect obtained on day 26 ($P<0.001$).

Figure 3C shows the effect of both extracts of *D. benthamianus* on arthritis scores. There was an increase in arthritis scores in CFA-induced rats from day 8, compared to the normal control group ($P<0.001$). The *D. benthamianus* extracts at all doses reduced

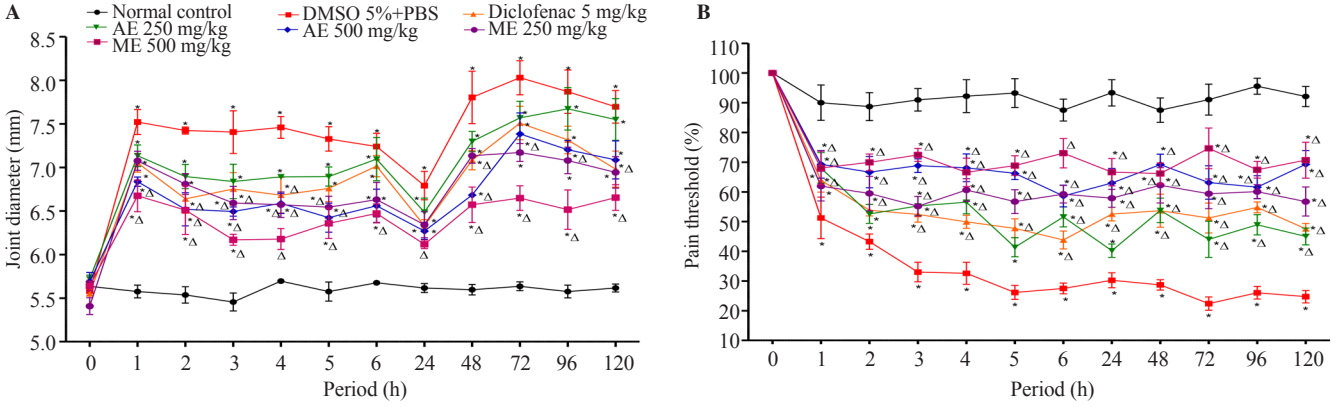

Figure 1. Effect of the aqueous (AE) and methanolic (ME) extracts of the stem bark of *Distemonanthus benthamianus* on joint diameter (A) and pain threshold (B) in zymosan A-induced monoarthritis rats. The data are expressed as mean±SEM and analyzed by two way ANOVA followed by Bonferroni *post-hoc* test. $^{*}P<0.05$ compared with the normal control group, $^{\Delta}P<0.05$ compared with the negative control group (DMSO 5% + PBS).

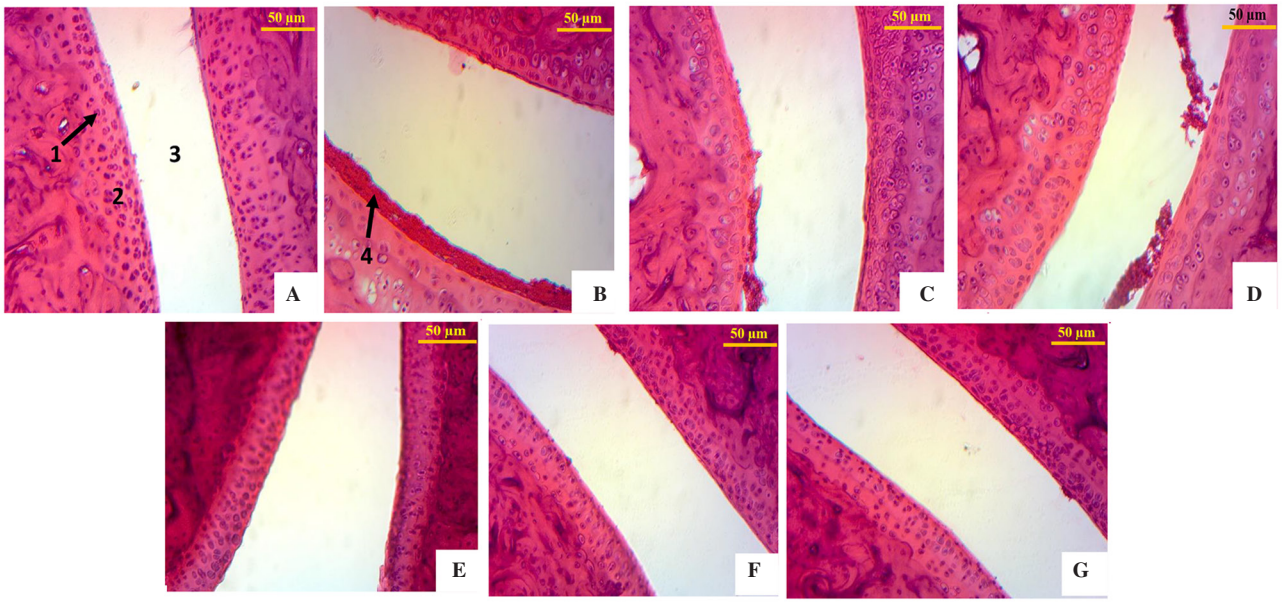

Figure 2. Microphotographs of the ankle joints 5 days after injection of zymosan A (hematoxylin-eosin; magnification: ×100). (A) The normal control group shows well-developed cartilage with proliferative chondrocytes. (B) The negative control group (DMSO 5%+PBS) shows that the cartilage is eroded with regressive chondrocytes and leukocyte infiltration, causing a large joint cavity. The diclofenac-treated group (C) and the groups receiving 250 and 500 mg/kg of aqueous extracts (D-E) or 250 and 500 mg/kg of methanolic extracts (F-G) show well-developed cartilage with proliferative chondrocytes. 1: chondrocytes; 2: articular cartilage; 3: joint cavity; 4: leukocyte infiltration.

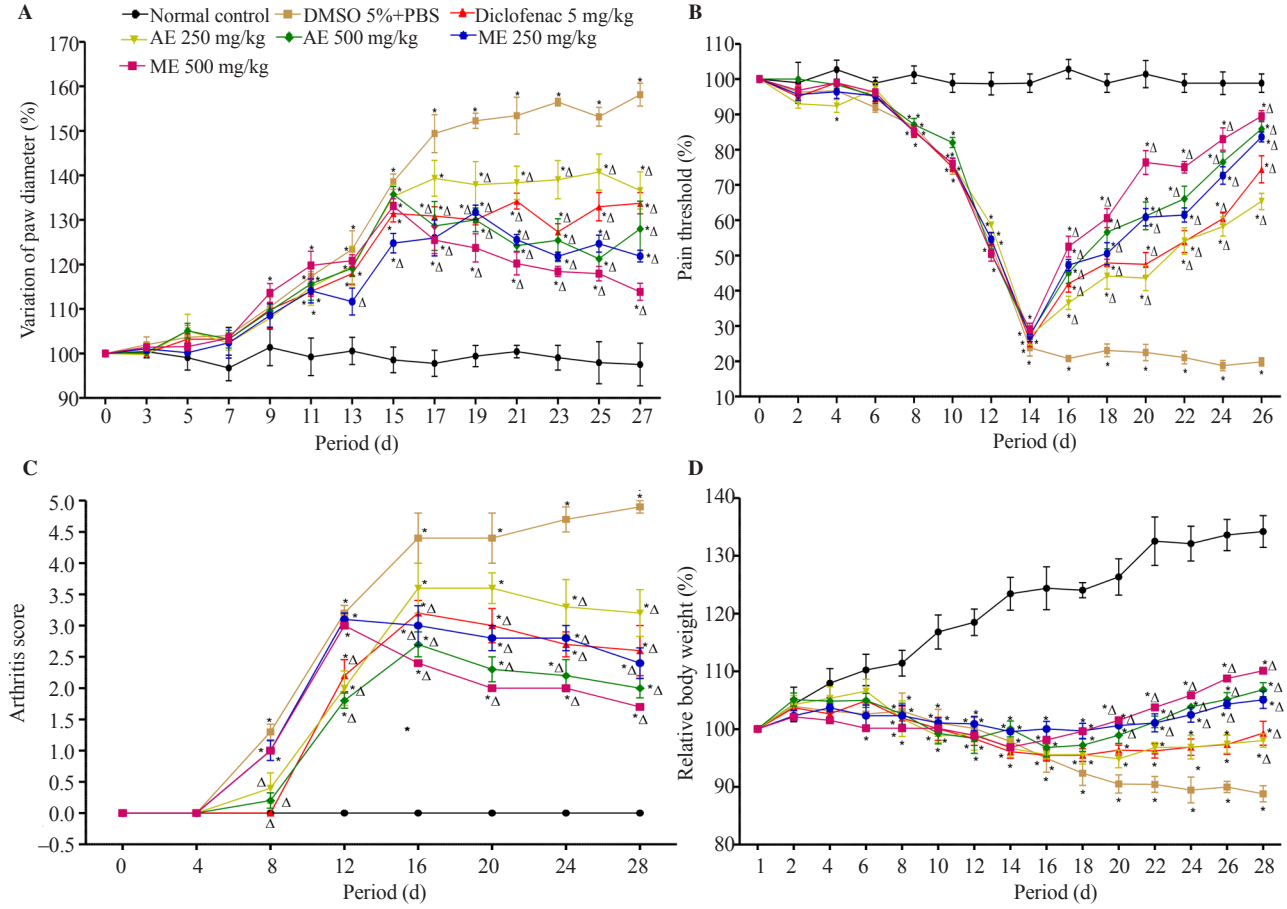

Figure 3. Effect of aqueous and methanolic extracts of *Distemonanthus benthamianus* on joint thickness (A), pain threshold (B), arthritis score (C) and body weight (D) after complete Freund's adjuvant (CFA) injection. The data are expressed as mean±SEM and analyzed by two way ANOVA followed by Bonferroni *post-hoc* test. $^{*}P<0.05$ compared with the normal control group, $^{\Delta}P<0.05$ compared with the negative control group (DMSO 5% + PBS).

the arthritis score in comparison with the negative control group (P<0.05).

3.2.2. Effect of extracts on body weight

Figure 3D illustrates the effect of *D. benthamianus* extracts on body weight. The weight of rats in the normal control group increased gradually during the treatment period. Conversely, CFA caused a significant decrease in body weight from day 8, compared to the normal control group (P<0.001). From day 20, rats treated with both extracts (500 mg/kg) or with diclofenac (5 mg/kg) showed significantly increased body weight (P<0.05).

3.2.3. Effect of D. benthamianus extracts on relative organs weight

Table 1 shows the effect of extracts on the relative weight of some organs 28 d following CFA administration. These results show an increase in weight of the liver, kidneys and spleen in the negative control group in comparison with the normal control group (P<0.001). Moreover, the weights of the thymus and lungs decreased in this group (P<0.01). Administration of *D. benthamianus* aqueous and methanolic extracts reduced the weights of the liver, kidneys, and spleen (P<0.05), but increased the weights of thymus and lungs compared to the negative control group.

3.2.4. Effect of D. benthamianus extracts on hematological parameters

The effects of extracts on some hematological parameters 28 d after CFA injection are shown in Table 2. The levels of rat blood platelets and white blood cells increased (P<0.001) while those of red blood cells, hemoglobin, and hematocrit significantly decreased (P<0.001) in the negative control group, in comparison with the normal control group. Furthermore, both extracts and diclofenac (5 mg/kg) prominently attenuated these changes, especially at the dose of 500 mg/kg (P<0.01).

3.2.5. Effect of D. benthamianus extracts on biochemical parameters

The effects of both extracts on biochemical parameters 28 d after CFA injection are summarized in Table 3. The activities of AST, ALT, and ALP were increased (P<0.001), while total protein concentration was decreased (P<0.01) in the negative control

Table 1. Effect of the aqueous and methanolic extracts of *Distemonanthus benthamianus* on the variation of organ weight after CFA injection (g).

Groups	Liver	Kidneys	Lungs	Spleen	Thymus
Normal control	6.16 ± 0.13	0.62 ± 0.03	1.48 ± 0.03	0.80 ± 0.03	0.66 ± 0.01
Negative control	9.03 ± 0.15***	1.01 ± 0.03***	1.05 ± 0.05**	1.32 ± 0.02***	0.40 ± 0.02***
Diclofenac (5 mg/kg)	7.08 ± 0.13$^{\Delta\Delta\Delta}$	0.76 ± 0.01$^{***\Delta\Delta\Delta}$	1.35 ± 0.07	0.92 ± 0.04$^{*\Delta\Delta\Delta}$	0.47 ± 0.02***
Aqueous extract (250 mg/kg)	7.47 ± 0.17$^{***\Delta\Delta\Delta}$	0.84 ± 0.02$^{***\Delta\Delta}$	1.14 ± 0.08*	1.16 ± 0.01$^{***\Delta}$	0.44 ± 0.03***
Aqueous extract (500 mg/kg)	6.79 ± 0.13$^{\Delta\Delta\Delta}$	0.65 ± 0.01$^{\Delta\Delta\Delta}$	1.36 ± 0.02	0.91 ± 0.01$^{\Delta\Delta\Delta}$	0.53 ± 0.02$^{*\Delta}$
Methanolic extract (250 mg/kg)	6.76 ± 0.32$^{\Delta\Delta\Delta}$	0.74 ± 0.01$^{**\Delta\Delta\Delta}$	1.23 ± 0.08	1.02 ± 0.04$^{***\Delta\Delta\Delta}$	0.48 ± 0.04***
Methanolic extract (500 mg/kg)	6.27 ± 0.28$^{\Delta\Delta\Delta}$	0.63 ± 0.02$^{\Delta\Delta\Delta}$	1.41 ± 0.07$^{\Delta}$	0.81 ± 0.01$^{\Delta\Delta\Delta}$	0.64 ± 0.01$^{\Delta\Delta\Delta}$

The data are expressed as mean±SEM and analyzed by one way ANOVA followed by Tukey *post-hoc* test. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 compared with the normal control group, $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01, $^{\Delta\Delta\Delta}P$<0.001 compared with the negative control group (DMSO 5% + PBS).

Table 2. Effect of the aqueous and methanolic extracts of *Distemonanthus benthamianus* on hematological parameters after CFA injection.

Groups	White blood cell (10^9/L)	Red blood cell (10^9/μL)	Hemoglobin (g/dL)	Hematocrit (%)	Platelet (10^9/L)
Normal control	7.03 ± 0.20	7.88 ± 0.76	16.75 ± 0.69	44.35 ± 2.06	518.80 ± 34.77
Negative control	12.50 ± 0.33***	3.94 ± 0.16***	8.72 ± 0.26***	24.34 ± 1.18***	896.60 ± 7.13***
Diclofenac (5 mg/kg)	9.96 ± 0.54$^{**\Delta}$	5.31 ± 0.42**	12.94 ± 0.54$^{***\Delta\Delta\Delta}$	35.60 ± 1.24$^{**\Delta\Delta\Delta}$	705.80 ± 11.93$^{***\Delta\Delta\Delta}$
Aqueous extract (250 mg/kg)	10.70 ± 0.45***	4.70 ± 0.31***	10.84 ± 0.22***	34.04 ± 1.59$^{***\Delta\Delta}$	797.80 ± 23.69$^{***\Delta}$
Aqueous extract (500 mg/kg)	9.24 ± 0.29$^{\Delta\Delta}$	6.67 ± 0.04$^{\Delta\Delta}$	13.78 ± 0.54$^{**\Delta\Delta\Delta}$	38.16 ± 1.51$^{\Delta\Delta\Delta}$	632.60 ± 22.70$^{*\Delta\Delta\Delta}$
Methanolic extract (250 mg/kg)	9.54 ± 0.44$^{*\Delta\Delta}$	6.57 ± 0.61$^{\Delta\Delta}$	12.82 ± 0.48$^{***\Delta\Delta\Delta}$	36.66 ± 0.96$^{*\Delta\Delta\Delta}$	636.20 ± 9.44$^{*\Delta\Delta\Delta}$
Methanolic extract (500 mg/kg)	8.24 ± 0.42$^{\Delta\Delta\Delta}$	7.49 ± 0.43$^{\Delta\Delta\Delta}$	14.84 ± 0.28$^{\Delta\Delta\Delta}$	40.48 ± 1.25$^{\Delta\Delta\Delta}$	579.00 ± 17.85$^{\Delta\Delta\Delta}$

The data are expressed as mean±SEM and analyzed by one way ANOVA followed by Tukey *post-hoc* test. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 compared with the normal control group, $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01, $^{\Delta\Delta\Delta}P$<0.001 compared with the negative control group (DMSO 5% + PBS).

Table 3. Effect of the aqueous and methanolic extracts of *Distemonanthus benthamianus* on serum parameters after CFA injection.

Groups	ALT (U/L)	AST (U/L)	Creatinine (mol/L)	ALP (U/L)	Total protein (g/dL)
Normal control	114.10 ± 1.01	433.90 ± 5.59	0.33 ± 0.02	228.80 ± 2.37	6.49 ± 0.23
Negative control	258.20 ± 3.97***	527.50 ± 6.92***	0.35 ± 0.01	390.20 ± 17.85***	4.93 ± 0.18**
Diclofenac (5 mg/kg)	156.60 ± 1.79$^{***\Delta\Delta\Delta}$	495.30 ± 15.51	0.31 ± 0.01	324.50 ± 4.76**	5.24 ± 0.24*
Aqueous extract (250 mg/kg)	188.70 ± 1.80$^{***\Delta\Delta\Delta}$	510.20 ± 17.37**	0.31 ± 0.03	341.70 ± 11.61***	4.10 ± 0.27***
Aqueous extract (500 mg/kg)	130.30 ± 0.39$^{\Delta\Delta\Delta}$	462.60 ± 11.57	0.32 ± 0.01	287.80 ± 25.23	5.58 ± 0.30
Methanolic extract (250 mg/kg)	124.40 ± 1.53$^{\Delta\Delta\Delta}$	471.20 ± 15.27	0.35 ± 0.01	265.60 ± 14.79$^{\Delta\Delta\Delta}$	5.17 ± 0.41*
Methanolic extract (500 mg/kg)	116.30 ± 2.52$^{\Delta\Delta\Delta}$	437.30 ± 13.87$^{\Delta\Delta\Delta}$	0.29 ± 0.03	252.50 ± 9.67$^{\Delta\Delta\Delta}$	6.25 ± 0.09$^{\Delta}$

The data are expressed as mean±SEM and analyzed by one way ANOVA followed by Tukey *post-hoc* test. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 compared with the normal control group, $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01, $^{\Delta\Delta\Delta}P$<0.001 compared with the negative control group (DMSO 5% + PBS).

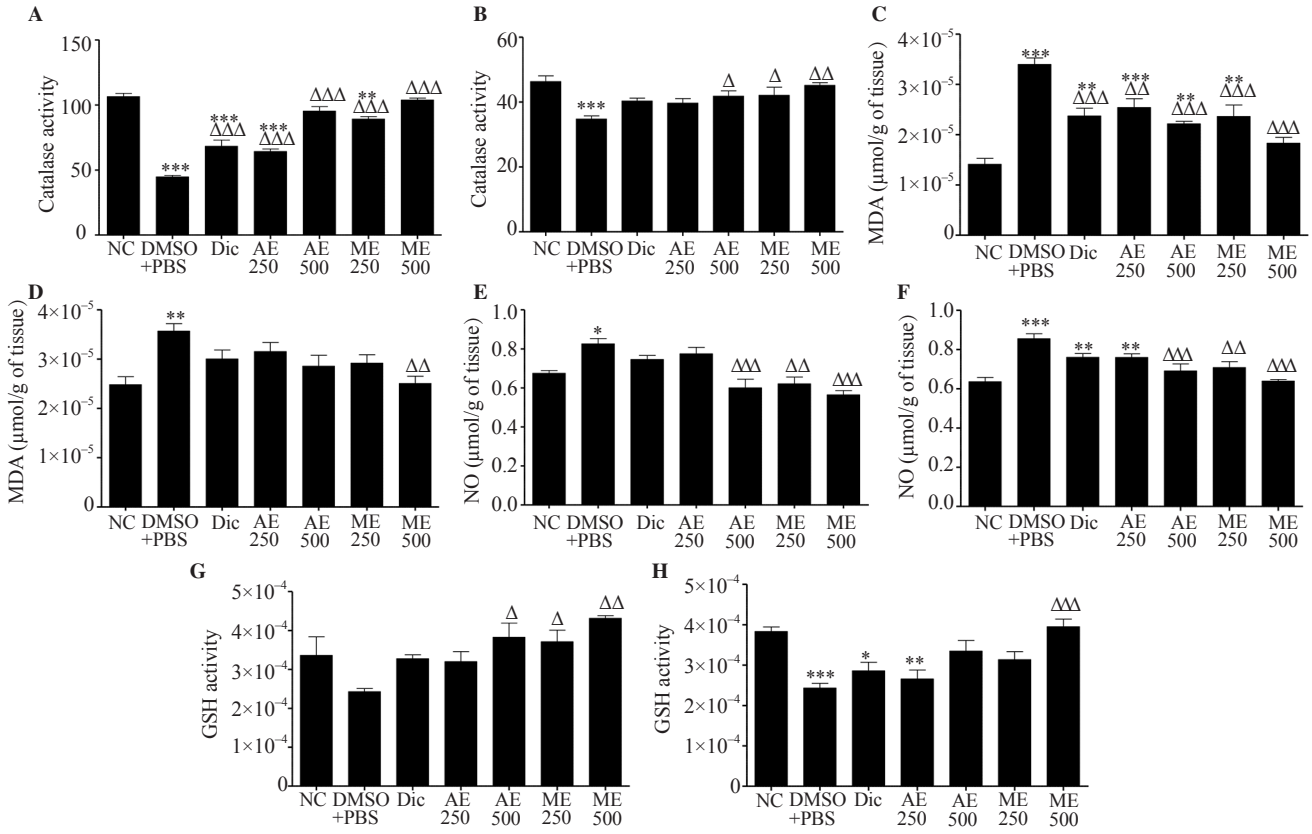

Figure 4. Effect of the aqueous and methanolic extracts of *Distemonanthus benthamianus* on the levels of oxidative stress markers. A: catalase; C: malondialdehyde (MDA); E: nitric oxide (NO); G: glutathione (GSH) in the liver and B: catalase; D: MDA; F: NO; H: GSH in the kidneys after CFA injection. The data are expressed as mean±SEM and analyzed by one way ANOVA followed by Tukey *post-hoc* test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the normal control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, $^{\Delta\Delta\Delta}P<0.001$ compared with the negative control group (DMSO 5% + PBS). Dic: Diclofenac; AE 250: Aqueous extract 250 mg/kg ; AE 500: Aqueous extract 500 mg/kg; ME 250: Methanolic extract 250 mg/kg ; ME 500: Methanolic extract 500 mg/kg.

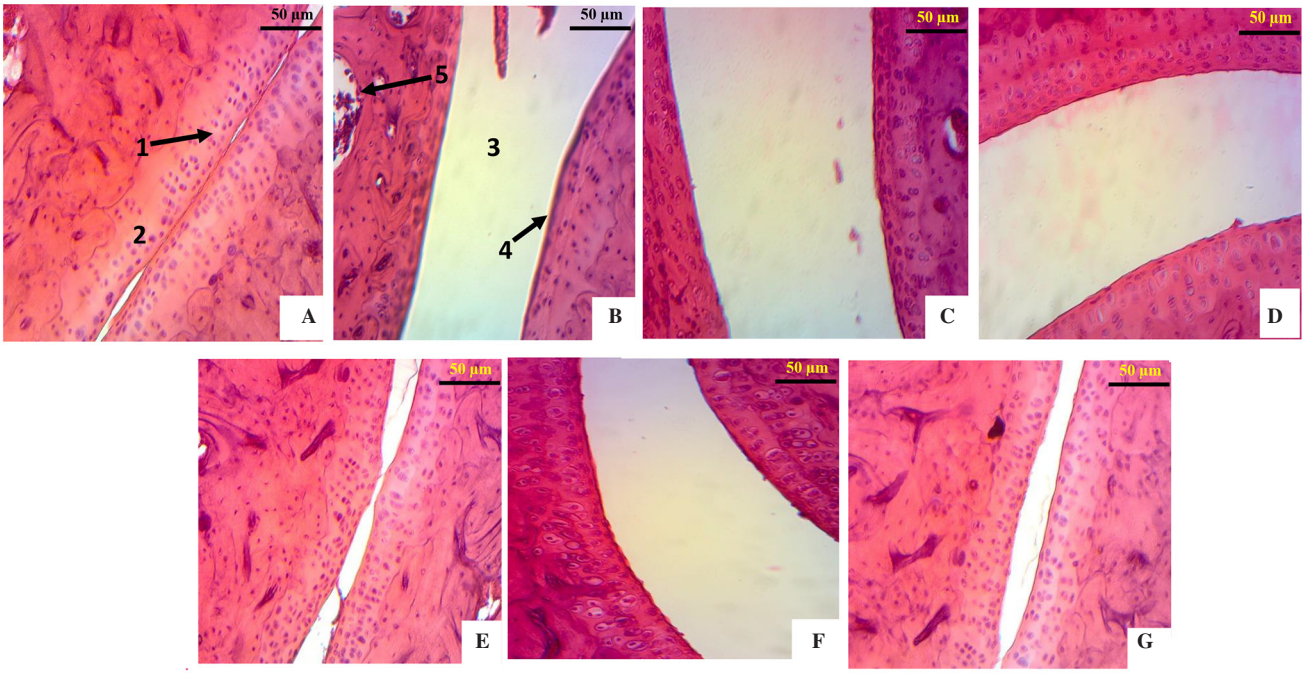

Figure 5. Microphotographs of the ankle joints (hematoxylin-eosin; magnification: ×100). A: The normal control group shows well-developed cartilage containing proliferative chondrocytes. B: The negative control group shows that the cartilage is eroded with regressive chondrocytes and leukocyte infiltration. The diclofenac-treated group (C) and the groups receiving 250 and 500 mg/kg of aqueous extracts (D-E) or 250 and 500 mg/kg of methanolic extracts (F-G) show well-developed cartilage with proliferative chondrocytes. 1: chondrocytes; 2: articular cartilage; 3: joint cavity; 4: leukocyte infiltration; 5: bone erosion.

group, when compared to the normal control group. The aqueous and methanolic extracts reduced ALT, AST, and ALP activities, while increasing the total protein levels, in comparison with the negative control group.

3.2.6. Effect of D. benthamianus extracts on oxidative stress markers

Figure 4 presents the effect of both extracts on oxidative stress markers 28 d after CFA injection. MDA and NO levels were markedly increased (P<0.05), while catalase and GSH levels decreased (P<0.001) in the negative control group. Moreover, 500 mg/kg of both extracts induced a significant decrease in MDA and NO levels, as well as a significant increase in catalase and GSH levels in comparison with the negative control group.

3.2.7. Effect of D. benthamianus on histological sections of the joints

Figure 5 illustrates the histological sections of joints. It was observed that rats of the negative control group (Figure 5B) showed a very large joint space and cartilage destruction with bone erosion and leukocyte infiltration. The joints of the positive control group and the groups treated with aqueous (250 and 500 mg/kg) and methanolic (250 and 500 mg/kg) extracts showed an unaltered synovial membrane, a significantly reduced joint space and intact cartilage with no leukocyte infiltration compared to the negative control group (Figures 5C-G).

4. Discussion

Results of the current study revealed that the aqueous and methanolic bark extracts of *D. benthamianus* had anti-arthritic activities in preventive treatment when testing their effects on zymosan A-induced monoarthritis and in curative treatment in rats with CFA-induced polyarthritis. It appears that the extracts significantly inhibited the clinical symptoms (swelling of the paw, pain threshold, decrease in body weight, arthritis score) and improved the biochemical, hematological, oxidative stress, and histological parameters that were disturbed after zymosan A or CFA injection.

Zymosan A, a polysaccharide from the cell wall of yeast (*Saccharomyces cerevisiae*) has the property of activating the cells of the innate immune system, therefore stimulating a local inflammatory response[27,28]. Thus, zymosan A injection into the rat joint provokes hypertrophy of the synovial membrane and infiltration of immune cells, which trigger the release of several pro-inflammatory mediators such as cytokines (TNF-α and IL-1), leukotrienes, prostaglandins, NO and ROS[28]. As a result, it follows a degradation of bone and joint cartilage[29] and inflammation of the synovial membrane[30] associated with a decrease in pain threshold. In this study, the aqueous and methanolic extracts of *D. benthamianus* significantly reduced joint diameter and increased pain threshold, which could be related to the inhibition of pro-inflammatory and pro-pain mediators' production and/or activity. Furthermore, histological analysis showed that rats receiving *D. benthamianus* extracts and diclofenac treatment showed less damaged cartilages and bones, no leukocyte infiltration, and a normal synovial membrane. The effects of these extracts on monoarthritis may be explained by the presence of different classes of compounds like flavonoids, terpenoids, saponins, alkaloids, and steroids. Several chemical compounds from these classes have proved their ability to reduce or treat the symptoms of RA[31]. In addition, we also used CFA-induced polyarthritis to further verify the anti-arthritic activity of the plant extract.

CFA-induced polyarthritis in rats is experimental modeling applied for preclinical testing of many anti-arthritic drugs, due to its close resemblances to human rheumatoid diseases[26]. CFA induces the release of a cascade of proinflammatory mediators such as cytokines and PGE_2[32]. These mediators are responsible for hypersensitization of nociceptors resulting in hyperalgesia. In this study, treatment with *D. benthamianus* extracts showed anti-arthritic effects. The plant extracts significantly inhibited inflammation *via* improving joint diameter, arthritis score, and hyperalgesia. Nevertheless, supplementary studies on pro-inflammatory mediators (cytokines, prostaglandins, leukotrienes) involved in arthritis pathogenesis are required, to attest to the correlation between the observed activities on macroscopic indexes and inhibition of pro-inflammatory and/or pro-pain mediators. Weight loss is an indicator of health in pathological conditions. In RA, weight loss is considered a result of appetite loss or a metabolic disorder caused by the inflammatory reaction[26]. The weight loss observed in untreated animals was regulated in animals receiving *D. benthamianus* extracts as well as the standard drug. This effect is linked to a corrective effect of the extracts which may be due to stimulated appetite and restored metabolism by treatment. Thus, further analysis that elucidates the regulatory influence of treatment with extracts on glucose, amino acids, and fatty acids metabolism as well as their capacity of stimulating orexigenic factor release will support this hypothesis and needs to be conducted in the future.

Transaminases and ALP are indicators of liver and kidney damage. Furthermore, it is known that ALP activity increases significantly in pathogenic cases of RA. In addition, the enzymatic level of transaminases is important in the release of biologically active compounds like bradykinin, during the inflammatory process[26]. These enzymes, which are released into the circulation during bone formation and resorption, are usually present in the area where there is erosion and are responsible for the release of certain pro-inflammatory mediators such as serotonin, bradykinin, and histamine[33]. In people with arthritis, Patel *et al*.[34] observed a significant increase in ALT level. In this study, rats with arthritis

presented a significant increase in ALP, AST, and ALT. The increase in these enzymes was significantly reduced in animals treated with *D. benthamianus* extracts.

It is known that free radicals can destroy the lipid membrane and proteins, act on DNA structure, and damage joint cartilage[35]. Neutrophils and macrophages produce ROS following CFA injection and these ROS contribute to the destruction of cartilage which causes an increase in lipid peroxidation and the reaction catalyzed by NO, as well as a decrease in antioxidant defense, leading to oxidative stress[36]. In the present study, the effects of *D. benthamianus* on oxidative stress parameters were evaluated and both extracts significantly decreased MDA and NO levels while increasing GSH and catalase activities. These results suggest that these extracts act on NO and MDA synthesis by inactivating the enzyme NO synthase. They also decreased lipid peroxidation and increased GSH and catalase activities.

In the case of patients with RA, several systemic changes are usually observed, such as an increase in white blood cells and blood platelets as well as a reduction in hematocrit, hemoglobin, and red blood cells. These changes are considered to be vital markers that can testify to the establishment of arthritis[34], expressing an alteration of the immune system in the course of the pathology. The aqueous and methanolic bark extracts of *D. benthamianus* significantly ameliorated the systemic variation of blood parameters, as evidenced by a decrease in the levels of white blood cells and platelets, and an increase in red blood cells, hemoglobin, and hematocrit. These effects suggest that the extracts could stimulate and/or activate immune cells. Histological analysis showed that rats administrated with extracts and diclofenac showed a significant reduction in cartilage erosion, bone destruction, and joint space with no leukocyte infiltration.

Considering these results, the anti-arthritic effects of *D. benthamianus* may be due to the antioxidant and anti-inflammatory activities of its secondary metabolites. Indeed, alkaloids, flavonoids, polyphenols, saponins, sterols, tannins, triterpenes, and phenols[10] identified in the extracts of *D. benthamianus* are known to possess antioxidant, anti-inflammatory, analgesic, and/or anti-arthritic potentials[37].

In conclusion, the aqueous and methanolic extracts of *D. benthamianus* showed anti-arthritic effects against zymosan A- and CFA-induced RA rats. The plant extracts reduced paw swelling, increased pain threshold, ameliorated clinical symptoms, improved biochemical, oxidative stress, and hematological parameters, and protected the joints against tissue erosion. Therefore, this plant could be a promising candidate for the treatment of RA diseases.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

MM, KEAH and AG designed the work. MM, KEAH, YNW, TEG, DNSF, FNZL, MMVM, ACF, MKYK, and NAE conducted the work and collected and analyzed the data. MM, YNW, AG, and AAD drafted the manuscript and revised it critically. All authors agreed to be accountable for all aspects of the work.

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