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Psidium guajava leaves extract decreased leukocytes and lymphocytes count in Complete Freund's Adjuvant-induced arthritis rats

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ABSTRACT

Background: *Psidium guajava* is one of the herbal plants that has demonstrated antioxidant and anti-inflammatory properties.

Objective: This study aimed to determine the effect of *P. guajava* leaves extract on leukocytes and lymphocytes in rats.

Methods: Fifty rats were divided into five groups. Complete Freund's Adjuvant (CFA) was employed to generate an animal model of arthritis. Group I (arthritis control) received CMC Na, group II (positive control) received dexamethasone 3 mg/kg BW, and groups III-V received 250, 500, and 750 mg/kg BW of the ethanol extract of *P. guajava* leaves orally. Total leukocyte counts were calculated on days 13 and 29, and on day 29, the histological joints were inspected.

Results: The *P. guajava* leaves extract decreased the number of lymphocytes and total leukocytes. Before and after treatment, the group receiving doses of 250 mg/kg BW and 750 mg/kg BW of the extract showed a significant decrease in leukocytes. With treatment of 250, 500, and 750 mg/kg BW of *P. guajava* leaves ethanol extract, the lymphocyte count was also reduced. The *P. guajava* leaves extract at 750 mg/kg body weight showed the greatest effectiveness in suppressing leukocytes and lymphocytes.

Conclusion: The extract of *P. guajava* leaves has the potential to be developed as an anti-inflammatory for arthritis.

Keywords: rheumatoid arthritis, Psdium guajava, leukocyte, lymphocyte, complete freund's adjuvant

Introduction

Arthritis is a chronic inflammatory disease characterized by inflammation of the synovium, hyperplasia of synovial fluid, and pannus tissue that attacks and destroys adjacent cartilage and bone [1]. The characteristics of this disease in the form of chronic inflammation can be obtained with the research model of Adjuvant Induced Arthritis (AIA) induced by *Mycobacterium tuberculli*, where AIA is used as an experimental model in developing rheumatoid arthritis studies. AIA has been employed as a model of chronic inflammation and has a close association with the pathophysiology of inflammation. AIA leads to joint swelling, infiltration of inflammatory cells, bone destruction, cartilage erosion, and functional abnormalities in rats [2]. Inflammation of the arthritis began 8 to 10 days after Complete Freund's Adjuvant (CFA) treatment and peaked 15 to 17 days later. Furthermore, it will recover spontaneously within 24 days [3].

The prevalence of arthritis in Indonesia is still relatively high. Consequently, it is necessary to develop alternative medicines for arthritis, including natural ingredients. *Psidium guajava* has been widely used as herbal medicine in Indonesia. Studies *in vitro*

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and *in vivo* indicate that plants contain compounds with anti-inflammatory and anti-rheumatoid arthritis activities. *P. guajava* leaves extract contains essential oils, flavonoids, triterpenoids, vitamin C, tannins, and phenolics [4]. The leaves are commonly used as anti-inflammatory [5,6], antioxidant [7], and hypolipidemic agents [8]. Quercetin-3-O-glucopyranoside and morin are the main antioxidant compounds in the leaves of *P. guajava* [9]. The ethanol extract of *P. guajava* leaves showed antioxidant activity with an IC_{50} value of 13.42 g/mL. *P. guajava* leaves extract has anti-inflammatory activity in carrageenan-induced rats [10]. *P. guajava* leaves ethanol extract has also been shown to be chronic anti-inflammatory [11].

The anti-inflammatory potential exists in the ethanol extract of *P. guajava* leaves, which could be developed as an anti-arthritis agent. In this study, we investigated the effect of *P. guajava* ethanol extract on the number of leukocytes in the blood and lymphocytes in the joint tissues.

Methods

P. guajava leaves extraction

The leaves of *P. guajava* were collected from Pabuaran, Purwokerto, and then dried in a 70°C oven. The leaves were powdered and macerated for 5x24 hours with ethanol 96% ethanol (Sigma, No 64175). The mixture was filtered, and the liquid obtained was evaporated using a rotary evaporator for 90 minutes at a temperature of 70-80°C. The viscous extract was obtained by evaporating the liquid over a water bath.

Advujant-induced arthritis in rats

Fifty male Sprague-Dawley rats weighing 130-150 grams and aged 2-3 months were obtained from the Pharmacology and Toxicology Laboratory of the Faculty of Pharmacy, Gadjah Mada University. Rats were divided into five groups randomly (n=10). Rats were induced with Complete Freund's Adjuvant (CFA) (Sigma, No F5881) 1 mg/mL on the right thigh on the first day (0.2 mL) and the fifth day (0.1 mL) [12]. This research has been approved by the Medical Ethics and Health Sciences Commission of Faculty of Medicine, Jenderal Soedirman University.

Rats were treated as follows: group I (arthritis model rats given CMC Na), group II (arthritis model rats given dexamethasone 3 mg/kg intraperitoneal on days 14, 16, 18, 20, 22, 24, 26, and 28), group III (arthritis model rats given ethanol extract of *P. guajava* leaves 250 mg/kg body weight orally on days 14 to 28), group IV (arthritis model rats given ethanol extract of *P. guajava* leaves 500 mg/kg body weight), and group V (arthritis model rats given ethanol extract of *P. guajava* leaves 750 mg/kg body weight).

Leukocyte count in the blood

Blood was drawn through the retroorbital plexus on day 13 (as pretreatment data) and day 29 (as post-treatment data). Four rats were picked from each group as samples. Blood samples were promptly tested at the Biofit Laboratory in Purwokerto for leukocyte count.

Determination of the number of lymphocytes in the tissue

On day 29, joint tissue was collected for observation and lymphocyte counting. Each treatment group was comprised of three rats. The tibiotarsal joint tissue exhibiting arthritic symptoms was sized to 1 x 1 x 0.5 cm. Tissue was placed in a container containing a 10% formalin solution (Sigma, No. HT501128). The preparations were stained with hematoxylin-eosin (HE) and then examined using a light microscope (Olympus CH-12). To determine the number of lymphocytes, we counted the number of lymphocytes in five different fields of view for every 100 leukocytes.

Data analysis

Changes in the number of leukocytes and lymphocytes were analyzed with SPSS ver 26.0. The number of leukocytes pre- and post-treatment were analyzed by paired t-test or Wilcoxon, while the number of lymphocytes in the tissue was analyzed using ANOVA with a 95% confidence level, followed by the least significant different test. Data were depicted in figure by using Prism Grappad 9.0 (San Diego, USA). $p < 0.05$ was considered significant.

Results

Adjuvant-induced arthritis model macroscopic observations

Before being induced with CFA, the rats were able to walk properly and did not exhibit any signs of edema. On day 1, 0.2 mL of CFA was injected intradermally into the thighs of rats, followed by 0.1 mL of CFA on day 5. After the second induction, all rats

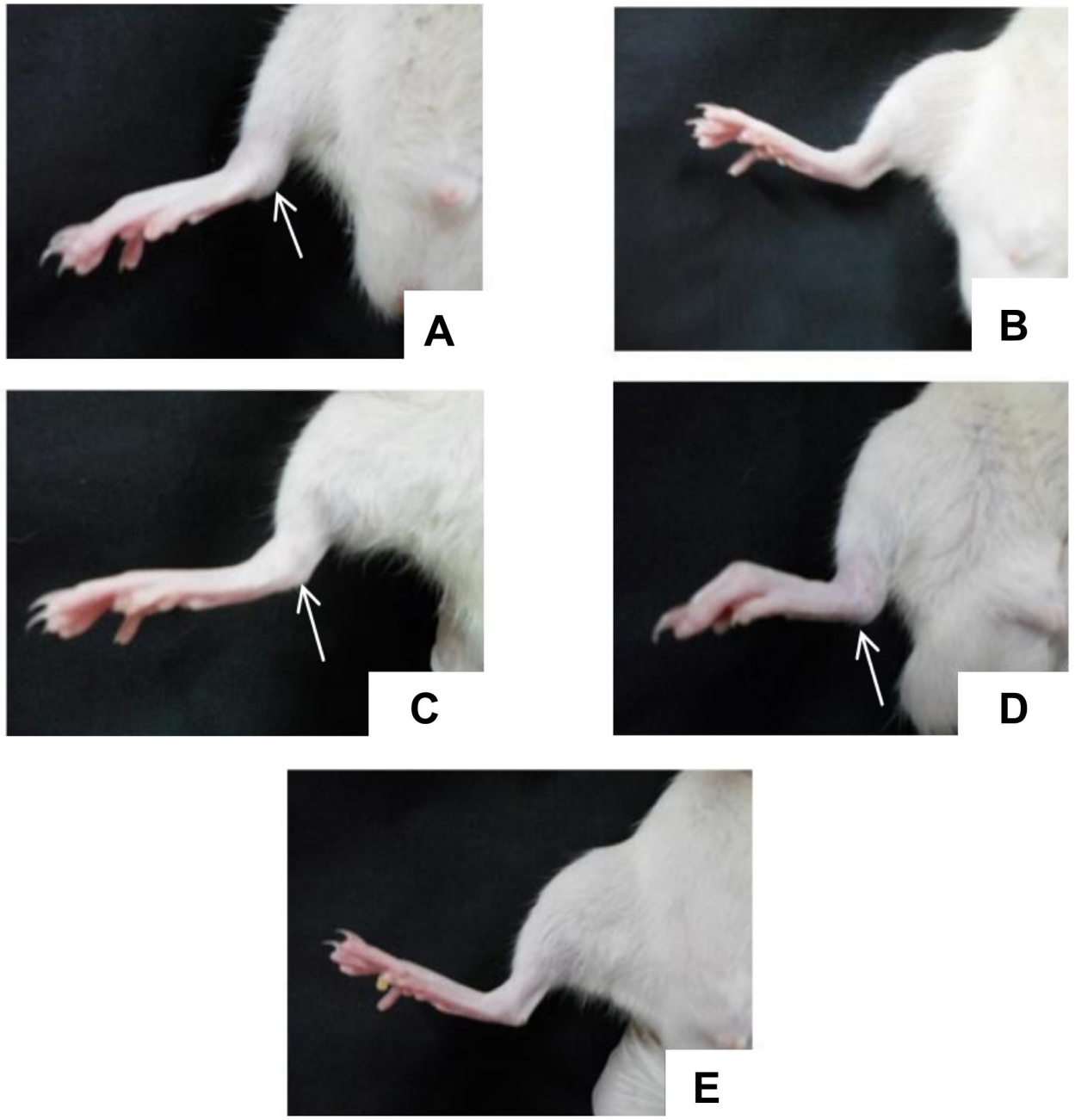

Figure 1. Joint appearance of rats on day 28. (A) arthritis control group (CFA), (II) Treatment of dexamethasone, *P. guajava* leaves ethanol extract group at a dose (C) 250 mg/kg BW, (D) 500 mg/kg BW, (E) 750 mg/kg BW in CFA-induced arthritis rats model. White arrows indicate swelling in the joint.

exhibited redness and swelling and could not walk with agility. In addition, edema was observed alongside the development of rheumatoid nodules on the ankles on day 28 in the arthritis group (Figure 1). This describes the clinical signs following CFA induction in terms of the clinical manifestations of arthritis. On day 28 after therapy, rats in the dexamethasone group, as well as those in all treatment groups with *P. guajava* ethanol extract, exhibited improvement.

Leukocyte count in the blood

Leukocytes in the blood of the test animals were counted on day 13 as pre-treatment data and day 29 as post-treatment data. The induction of CFA increased leukocyte accumulation in the blood of rats on day 13. In the CFA-induced arthritic group, the pre-treatment and post-treatment leukocyte count did not decrease significantly. In arthritis-model rats, treatment of dexamethasone, *P. guajava* leaves extract doses of 250

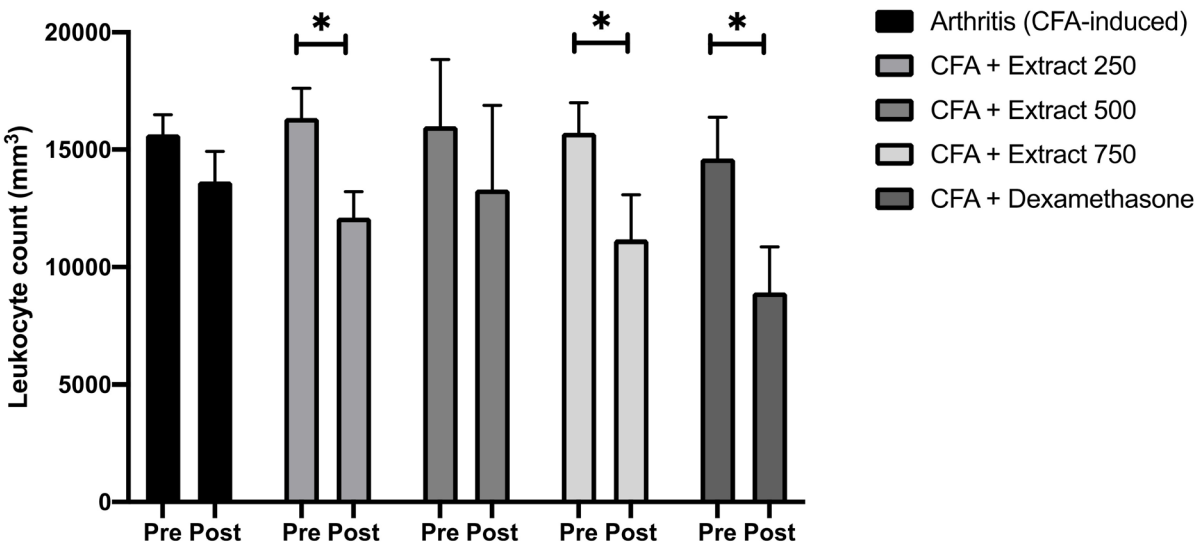

Figure 2. Leukocyte count pre- and post-treatment of *P. guajava* leaves ethanol extract in CFA-induced arthritis model. *p < 0.05

mg/kg BW and 750 mg/kg BW resulted in a significant reduction between pre-treatment and post-treatment, namely 39%, 26%, and 29%, respectively (Figure 2).

Lymphocyte count in tissues

The tibiotarsal joint in the right leg of necropsied rats was analyzed histopathologically to determine the number of lymphocytes in tissues (Figure 3). The dexamethasone and extract group had a reduced lymphocyte population than the arthritic control group ($p<0.05$). Dexamethasone and *P. guajava* leaves extract (250 mg/kg BW, 500 mg/kg BW, 750 mg/kg BW) treatment had a lymphocyte population of 29, 33, 26, and 27 cells per 100 cells, respectively (Figure 4), indicating that both dexamethasone and *P. guajava* leaves extract have anti-inflammatory activity. The extract dose of 750 mg/kg BW exhibited the greatest reduction in lymphocyte count.

Discussion

The induction of CFA provides the same clinical manifestations as arthritis. The clinical manifestation of AIA is inflammation of the joints and soles of the rat's feet. Inflammation is also characterized by the increase of leukocytes. Leukocytes that have accumulated in the circulation migrate to the tissues. The inflammatory process proceeds in the presence of these leukocytes' generated cytokines [13].

After treatment of *P. guajava* leaves extract, the number of leukocytes in CFA-induced rats decreased, indicating the anti-inflammatory effect of this extract. Oral administration of *P. guajava* ethanol extract produces anti-inflammatory effects [5]. The presence of leukocyte accumulation in rat blood indicates an inflammatory response. In a previous study, the number of leukocytes began to rise during the acute phase, and intraperitoneal administration of *P. guajava* leaves extract significantly decreased the number of leukocytes [14].

P. guajava leaves extract contains numerous essential oils, flavonoids, triterpenoids, vitamin C, tannins, and phenolics [9]. A decrease in leukocytes in the blood is suggested because the flavonoids in *P. guajava* extract inhibit leukocyte immobilization, thereby reducing leukocyte migration and adhesion molecules. Leukocyte immobilization is responsible for the formation of free radicals, the release of cytotoxic oxidants, and the activation of the complement system, which causes the release of inflammatory mediators and tissue damage [15].

The population of lymphocytes in the connective tissue of rats appeared after Mycobacterium injection, by which Bhsp65 antigen was captured by dendritic cells and carried to the lymph node, to be processed and presented to T lymphocytes [16]. T lymphocytes have receptors that identify the epitope of Bhsp65, allowing them to be activated and differentiated into cytokine-producing effector lymphocytes. Bhsp65-expressed CpG oligodeoxynucleotide may contribute to the biological action of CFA. Unlike mammalian DNA, bacterial DNA, including that of Mycobacterium, contains unmethylated

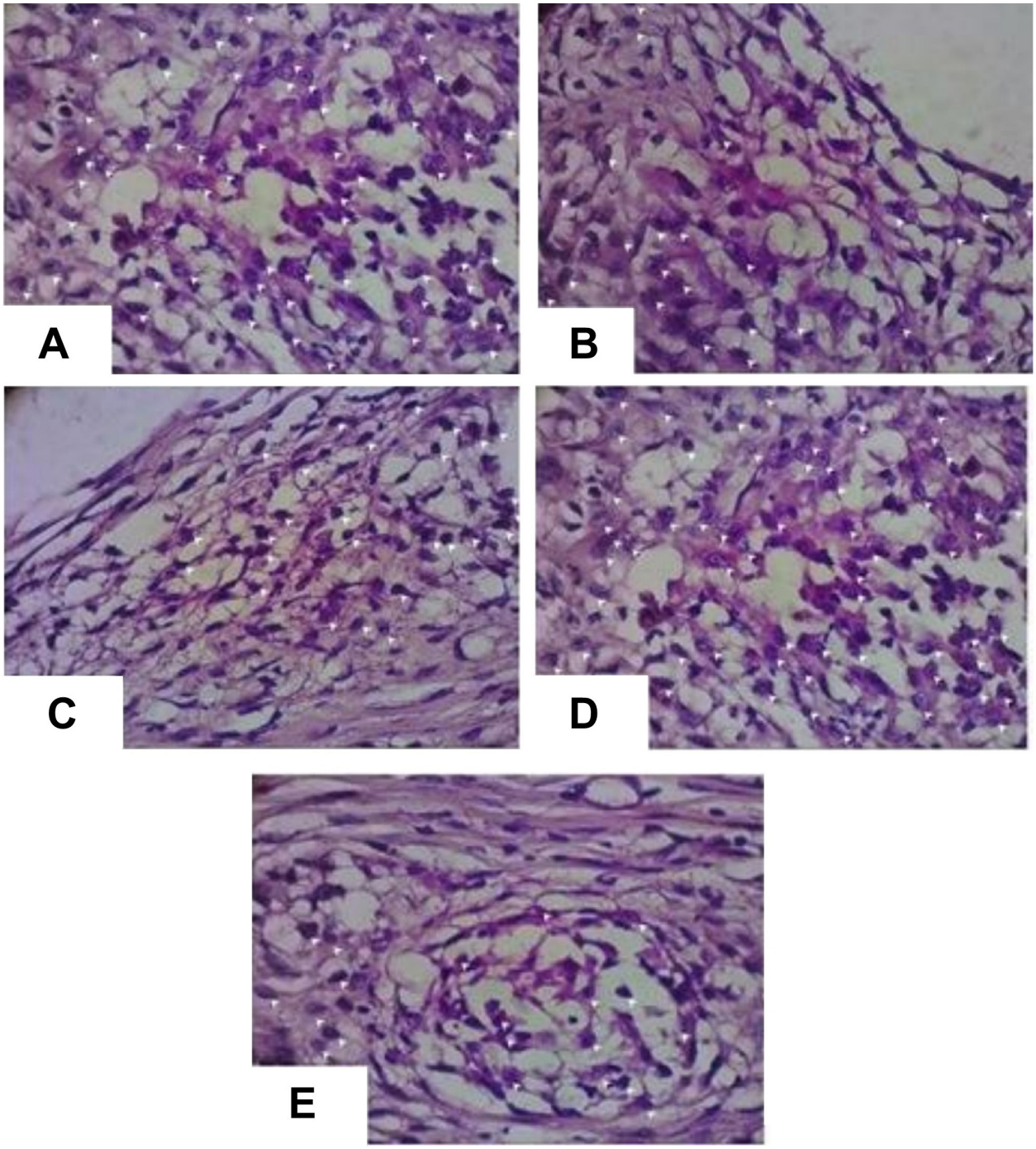

Figure 3. Histopathological image of the connective tissue of the tibiotarsal joint. (A) Arthritis control group (CFA-induced). Treatment of (B) dexamethasone, *P. guajava* leaves ethanol extract group at (C) 250 mg/kg BW, (D) 500 mg/kg BW, (E) 750 mg/kg BW in CFA-induced arthritis rats model. White arrows indicate lymphocytes. HE staining, 100x magnification

CpG dinucleotide. CpG oligodeoxynucleotide activates the innate immune system by stimulating the synthesis of cytokines, maturation, and activation of antigen presenting cells and Th1 cells [17].

T lymphocytes then migrate from the blood vessels to the target tissue, namely the joints, where Bhsp initiates the pathological process of arthritis as a result of cross-reactivity between the Mycobacterium epitope and the host epitope, thus explaining the increase in the lymphocyte population in arthritis [16]. Controlling the function and proliferation of lymphocytes is essential for maintaining the homeostasis of the immune system. Loss of this equilibrium results in uncontrolled proliferation or excessive decrease of

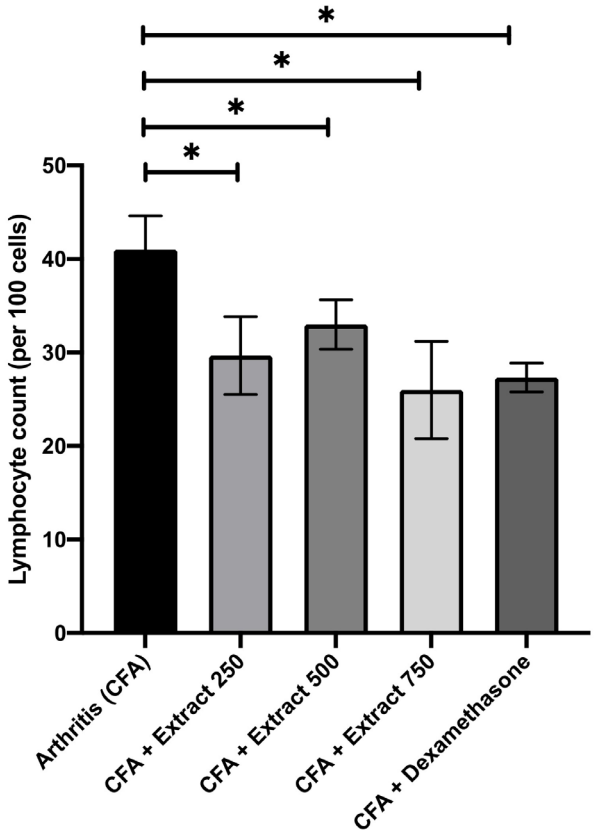

Figure 4. Lymphocyte count after treatment of *P. guajava* leaves ethanol extract in CFA-induced arthritis model. *p < 0.05

lymphocyte responses associated with autoimmunity. The production of NO at high concentrations by macrophages induces apoptosis in numerous cells and reduces the response of lymphocytes to antigenic stimuli, which can ultimately result in the impairment of immune system homeostasis [18].

The ethanol extract of *P. guajava* leaves possesses anti-inflammatory properties according to the number of leukocytes in rat blood and lymphocytes in joint tissue. The extract dose of 750 mg/kg BW had the best activity in reducing the number of leukocytes in the blood and lymphocytes in the tissues. Further research is needed to determine the role of *P. guajava* leaves extract at the molecular level, such as determining the number of inflammatory cytokines and critical regulatory genes in inflammation. Therefore, the ethanol extract of *P. guajava* leaves could be developed as an anti-arthritis agent.

Conclusion

The ethanolic extract of *P. guajava* decreases the number of leukocytes in the blood and the number of lymphocytes in the tissue of rats with CFA-induced arthritis. The effective dose of *P. guajava* leaves to reduce the number of leukocytes in the blood and the number of lymphocytes in the tissue of rats with CFA-induced arthritis was 750 mg/kg BW.

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Author contributions

HNB, EDU design the study; HNB, EDU, RL contribute to data acquisition; HNB, RL write the first draft; HNB, RL, NAZ performed analytical statistic; HNB, NAZ finalize and revise final manuscript; all authors agree to the last version manuscript.

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