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Research Article

Anti-Rheumatoid Arthritis Activity of 96% Ethanol Extract of *Eleutherine bulbosa* Bulbs with Arthritis Induction Adjuvant Method

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Abstract

An autoimmune condition known as rheumatoid arthritis (RA) results in chronic joint inflammation. Side effects that occur during long-term RA treatment are dangerous. Therefore, many people prefer herbal medicines, estimated to have lower side effects; one such herb is bawang dayak (*Eleutherine bulbosa* Urb.) bulbs. This study aimed to determine the class of compounds and the effective dose of the 96% ethanol extract of *E. bulbosa* bulbs, which had an anti-RA effect in the Wistar strain with the Adjuvant Induced Arthritis (AIA) model. *Eleutherine bulbosa* bulb extract was macerated with 96% ethanol. In the tests with extract doses of 100, 200, and 400 mg/KgBW and methylprednisolone 15 mg/KgBW, the induction used Complete Freund's Adjuvant (CFA). Treatment was provided from day eight through 21 of the test's 21-day duration. Phytochemical screening results contain alkaloids, flavonoids, phenols, quinones, saponins, steroids, and tannins. The percentage inhibition of edema volume and joint thickness, respectively, extract doses of 100, 200, 400 mg/KgBW, and methylprednisolone 15 mg/KgBW were 27.9585%, 49.3446%, 53.3239%, and 58.4629%; as well as 64.9809%, 73.8022%, 74.1444%, and 74.1825%. After analyzing the results, it was determined that *E. bulbosa* bulb extracts in 96% ethanol can treat RA at effective 200 and 400 mg/KgBW (p-value <0.05).

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INTRODUCTION

An autoimmune disease called rheumatoid arthritis (RA) results in chronic inflammation of the joints because the immune system of the affected joint accidentally attacks the body's tissues[1]. According to WHO[2], the number of RA sufferers in the world is 30.8-35.4%. Joint disease in Indonesia in 2013 was 24.7% based on the diagnosis and symptoms experienced[3]. Side effects that occur during long-term RA treatment are dangerous. The things that can happen are gastrointestinal bleeding, nausea, dyspepsia, impaired kidney function, increased blood pressure, headaches, and dizziness[4]. Therefore, many people prefer herbal medicines that have lower side effects.

Bawang dayak (*Eleutherine bulbosa* Urb.) bulbs are one of the plants that have the potential to be antiinflammatory, antiviral, and antibacterial, and they contain eleutherine and isoeleutherine compounds[5,6]. The 96% ethanol extract of *E. bulbosa* has an antiinflammatory effect when tested *in vitro* on the stability of human red blood cell membranes (HRBC) resulting in an EC_{50} value of 52.87%[7]. The methanol extract of *E. bulbosa* bulbs on the HRBC membrane stability has an antiinflammatory activity of 72.74%[8]. At *in vivo* activity against inflammatory cells of the dental pulp of Wistar rats, the effectiveness of 40% extract of *E. bulbosa* bulbs reduced the number of inflammatory cell infiltrates (neutrophils, macrophages, and lymphocytes)[9]. In addition, *in vivo* antiinflammatory tests 70% ethanol solvent at 240 mg/0.1 kgBW showed antiinflammatory activity[10].

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Eleutherine bulbosa bulbs contain napthoquinonens and their derivatives, among them eleutherin, isoeleuherol, and eleutherin. Other ingredients include alkaloids, flavonoids, glycosides, phenolics, saponins, triterpenoids, steroids, and tannins[11]. Ingredients that have antiinflammatory effects are quinones and their derivatives[12]. The results of research[8] stated that the chemical content of flavonoids, phenols, and tannins are known to act as antiinflammatories, and another research[13], the presence of steroids, flavonoids, and alkaloids are substances that have anti-rheumatoid arthritis, stated that produce anti-rheumatoid arthritis activity.

Based on the antiinflammatory test data and the content of compounds that affect these activities, further research tests were carried out for *E. bulbosa* bulbs as anti-rheumatoid arthritis using the Adjuvant Induced Arthritis (AIA) method. This method is an immune-mediated model of chronic arthritis that incorporates intradermal injection with Complete Freund's Adjuvant (CFA) containing *Mycobacterium* or bacterial wall[14]. Complete Freund's Adjuvant inducers provide a clinical picture similar to RA states and pathogenesis with naturally occurring RA conditions. Complete Freund's Adjuvant was more accessible and affordable than other RA inducers[15].

MATERIALS AND METHODS

Materials

The tools were analytical balance (Ohaus®), macerator, micropipette (Socorex), oven (Memmert UN55), plethysmometer, rotary evaporator (IKA-RV 10 basic), stopwatch, thermometer, vortex, and waterbath. The sample was *E. Bulbosa* bulbs obtained from Landasan Ulin District, Banjarbaru, South Kalimantan, Indonesia, in December 2020. The collected plant was determined at the Herbarium Bogoriense, Indonesian Institute of Sciences, Bogor, with number 2242/IPH.1.01/If.07/XII/2019, and the result showed the plant included Iridaceae family with species name *Eleutherine bulbosa* Urb., as presented in **Figure 1**. The other materials used were distilled water, amyl alcohol, ammonia, cocktail rat anesthetic, concentrated hydrochloric acid, sulfuric acid, acetic acid, CFA (Sigma®), 96% ethanol, gelatin, chloroform (Merck), iron (III) chloride solution, Na-CMC 0.9%, sodium chloride, diclofenac sodium, Dragendorff's reagent, Mayer's reagent, Wagner reagent, magnesium powder, and methylprednisolone.

Figure 1. *Eleutherine bulbosa* bulbs.

Methods

Simplicia preparation

The simplicia was gathered, sorted, and thoroughly cleaned using running water. Next, the sample was chopped and left dry in the sun with a black cloth covering it. Then, the sample was sorted again, mashed, and sieved using a no.40 mesh[16].

Extraction

The procedure used maceration with 96% ethanol (1 sample : 3 solvent). Maceration was carried out for 24 hours and occasionally stirred for the first six hours. The remaceration process was repeated twice. The maserate was evaporated with a rotary evaporator at 40 rpm, 50ºC. The extract was concentrated on a waterbath at 50ºC until a thick extract[17].

Pytochemical screening[18-20]

Alkaloid tests: As much as 0.5 g of the sample was diluted in 15 mL of distilled water, then 5 mL of HCl was added and filtered. The filtrate was then split into three parts.

1. Wagner's test: As much as 3 mL of filtrate was added with 3-5 drops of Wagner reagent. Alkaloids were present when brown or reddish precipitate forms.
2. Mayer's test: As much as 3 mL of filtrate was added with 3-5 drops of Mayer reagent. Alkaloids were present when a white or yellowish precipitate forms.
3. Dragendorff's test: As much as 3 mL of filtrate was added with 3-5 drops of Mayer reagent. Alkaloids were present when the precipitate turned brick red.

Phenolic test: Fifteen mL of distilled water was used to dissolve the 0.2 g sample. Then heated for 10 minutes and filtered. The filtrate was then referred to as solution A. 2-3 drops of a 5% $FeCl_3$ solution were added to 5 mL of solution A. The presence of phenols was indicated by colors such as green, blue-green, or bluish-black.

Flavonoid test: Five mL of solution A was added to magnesium powder, 2 mL amyl alcohol, and 2 mL HCl. The mixture was shaken vigorously and then allowed to separate, and the color formed on the amyl alcohol layer was observed. The formation of a red, yellow, or orange color on the amyl alcohol layer indicates the presence of a group of flavonoid compounds.

Quinone test: Five mL of solution A was added with a few drops of 1 N NaOH solution, and the formation of a red color indicated the presence of a group of quinone compounds.

Saponin test: Five mL of solution A was put into a test tube, shaken for 10 seconds, and allowed to stand for 10 minutes, then added with 2-3 drops of 2 N HCl. A stable foam formed indicated the presence of saponin group compounds.

Steroid and triterpenoid test: Five mL of solution A was dissolved with 2 mL of chloroform in a test tube, then 10 drops of acetic anhydride were added. The solution was added with three drops of concentrated H_2SO_4 through the wall of the test tube. If the result was a brownish or violet ring on the boundary of the two solvents, it indicates the presence of triterpenoids, while the form of a green color indicates the presence of steroids.

Tannin test: Five mL of solution A was added with 10 drops of 1% gelatin solution. The formation of a white precipitate indicates the presence of a tannin group compound.

Anti rheumatoid arthritis test

Ethical clearance was obtained from the Medical and Health Research Ethics Commission, Faculty of Medicine, Universitas Surabaya, with No: 140/KE/X/2020. The extract doses were 100, 200, and 400 mg/KgBW. Methylprednisolone was used as a positive control. The test animals were given anesthesia before the antirheumatoid arthritis effect was induced. The anesthetic used was a mixture of 100 mg/mL ketamine, 20 mg/mL xylazine, and 10 mg/mL acepromazine with a dose of 0.2 mL/200 g/BW administered intramuscularly. The group of test animals was carried out randomly, with five treatment groups and five test animals in each group. Assessment of the anti-rheumatoid arthritis effect was carried out *in vivo* using female Wistar rats aged 6-8 weeks. The three-phase induction, treatment, and evaluation phases were used to assess anti-rheumatoid arthritis animal models[21,22].

Induction phase: Adjuvant induced arthritis (AIA), which Woode modified, was used to initiate RA. On the rat's left foot, 0.1 mL of Complete Freund's adjuvant (CFA) was intraplantarly injected into each test animal. Complete Freund's adjuvant of *Mycobacterium tuberculosis* antigen at a 1 mg/mL suspension concentration. The CFA suspension was homogenized with a vortex before injection. Before the injection, the weight of the rats was weighed, and the volume of edema as well as joint thickness were measured.

Treatment phase: Based on the clinical signs of arthritis and the anti-inflammatory response, the test extract was given on the eighth day. The chronic phase of RA, which appeared after the acute phase (days 4-5), was what caused the disease's clinical

symptoms, such as an increase in joint thickness and edema volume. On the eighth day, the animals were randomly divided into five groups. The 0.5% Na-CMC was the negative control group (NCG), while 15 mg/KgBW of methylprednisolone was the positive control group (PCG). The treatment group received 100, 200, and 400 mg/KgBW extract doses, respectively. For 14 days, it was administered as the test solution every day since it was grouped.

Evaluation phase: Evaluation was carried out to determine the effectiveness of the treatment and the evolution of the test environments for the treated animals. The evaluation was carried out by observing the test animals' edema volume and joint thickness. During the assessment of the antirheumatoid arthritis effect, edema volume and joint thickness were observed every day. The volume total of edema and joint thickness on days 8 to 21 for each group were determined in arbitrary units to determine the Area Under the Curve (AUC) value and the percentage of inhibition. The calculations were in the **Equations 1** and **2**, in which Vtn_{-1} was the edema volume average in tn_{-1}, Vtn was the edema volume average in tn, the AUC control total was the AUC total of the edema volume average against the time curve for the negative control, and the AUC Test total was the AUC total of edema volume average against the time curve for the treatment group/test animals.

$$\mathrm{AUC}_{tn-1}^{tn} = \frac{\mathrm{Vtn}-1+\mathrm{Vtn}}{2}\ (tn - tn - 1) \quad [1]$$

$$percentage\ of\ inhibition = \frac{\text{AUC control total}-\text{AUC test total}}{\text{AUC control total}} \text{x} 100\% \quad [2]$$

Data analysis

The first data analysis of the research used the Shapiro-Wilk test and the Test of Homogeneity of Variances, followed by a non-parametric test using the Kruskal-Wallis test, to determine whether there were significant differences between two or more data groups.

RESULTS AND DISCUSSION

The sample was extracted using the maceration method with the aim that there was not enough heat, which was feared to damage the active metabolite compounds[23]. The extract yield percentage was 4.87%. Phytochemical screening at the test reaction showed the form of color or precipitate as the reaction of metabolites in plants with reagents. One of the metabolite compounds that have an anti-inflammatory mechanism is flavonoids. Flavonoids inhibit cyclooxygenase (COX), lipoxygenase (LOX), degranulation, neutrophil, and histamine. Additionally, it prevents the release of lysosomal and endothelial enzymes, which causes the inflammatory process to proliferate and exude[24]. Arachidonic substrate for the cyclooxygenase route and the lipoxygenase pathway is inhibited as a result of the inhibition of arachidonic acid release[25,26]. Pytochemical screening results showed at **Table I**.

Table I. Phytochemical screening.

Compounds	Reagents	Results	Information
Alkaloids	Wagner's	+	Brown/reddish precipitate
	Mayer's	+	Red-yellowish precipitate
	Dragendorff's	+	Brick red precipitate
Phenols	5% ferri (III) chloride	+	Bluish green
Flavonoids	Mg powder + hydrochloric acid + amyl alcohol	+	Formation red-orange color on amyl alcohol layer
Quinones	1 N NaOH	+	Formation of a red color
Saponins	Aquadest	+	Formation of a stable foam
Steroids	Acetic anhydride + H_2SO_4	+	Formation of a green color
Taninns	1% gelatin solution	+	Formation of a precipitate

Before treatment, the test animals were anesthetized which would have an effect after 10-15 minutes of administration, shown by the test animals being slightly weakened—the anesthetic mechanism of action as sedation and hypnosis. After administration of CFA injection, days 3-5 showed an acute inflammation phase. With the AIA method, CFA induction closely resembled human rheumatoid disease and was commonly utilized to assess inflammatory illness. It was reliable as a model for persistent pain and an experimental model of CFA-induced polyarthritis in animal tests[27]. The arthritis

macroscopic scoring parameters were used as indications that test animals had rheumatoid arthritis, which is shown in **Figure 2**.

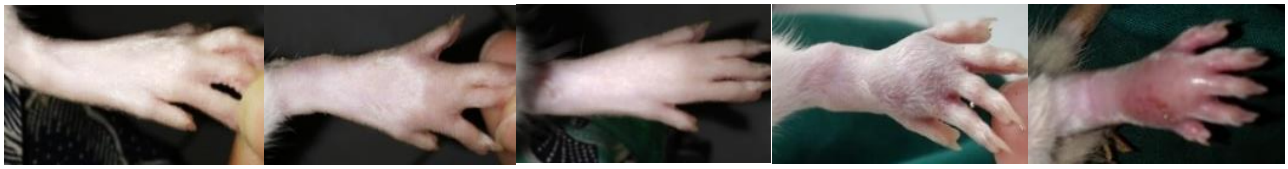

Figure 2. Mouse paw of **a**) normal condition, **b**) swelling or redness in one joint/muscle, **c**) swelling or redness in two joints/muscles, **d**) swelling or redness in more than two joints/muscles, and **e**) swelling or redness at all-paw, severe arthritis.

The positive group was given methylprednisolone 15 mg/KgBW because it belongs to the class of corticosteroids that can treat RA. The mechanism of methylprednisolone as an anti-inflammatory inhibits the synthesis of arachidonic acid, so it didn't form prostaglandins and leukotrienes to release inflammatory mediators and reduce vascular permeability in inflamed areas[28]. The edema volume in the paw and joint thickness were measured from days 0 to 21. Since the treatment phase started (day 8th), both with the administration of extracts and positive control, there was a decrease in edema volume and joint thickness. The results of the edema volume and joint thickness obtained were summed to get the AUC value. **Figure 3** shows the AUC of edema volume, and **Figure 4** shows the AUC of joint thickness of each treatment.

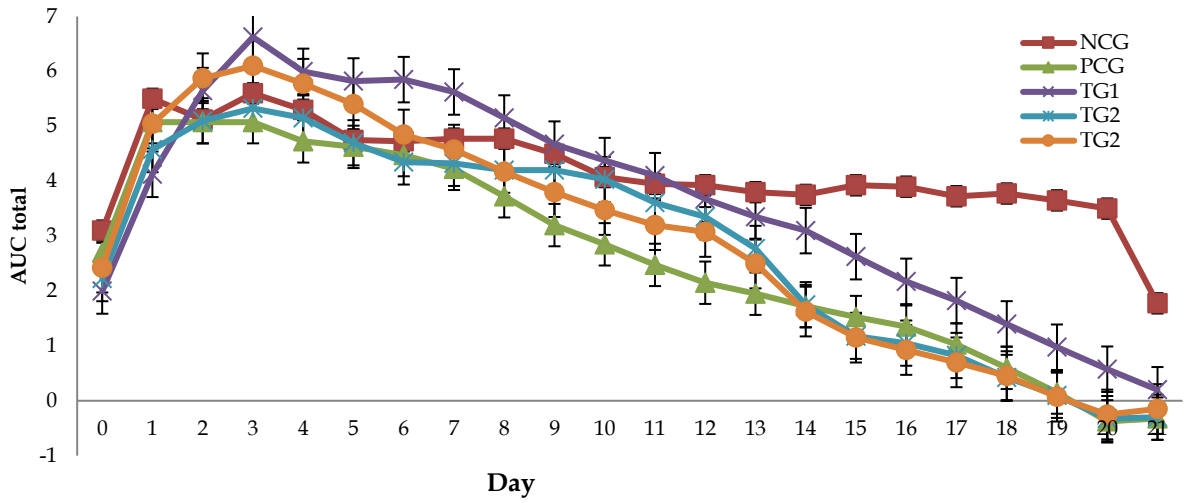

Figure 3. Total AUC value of edema volume.

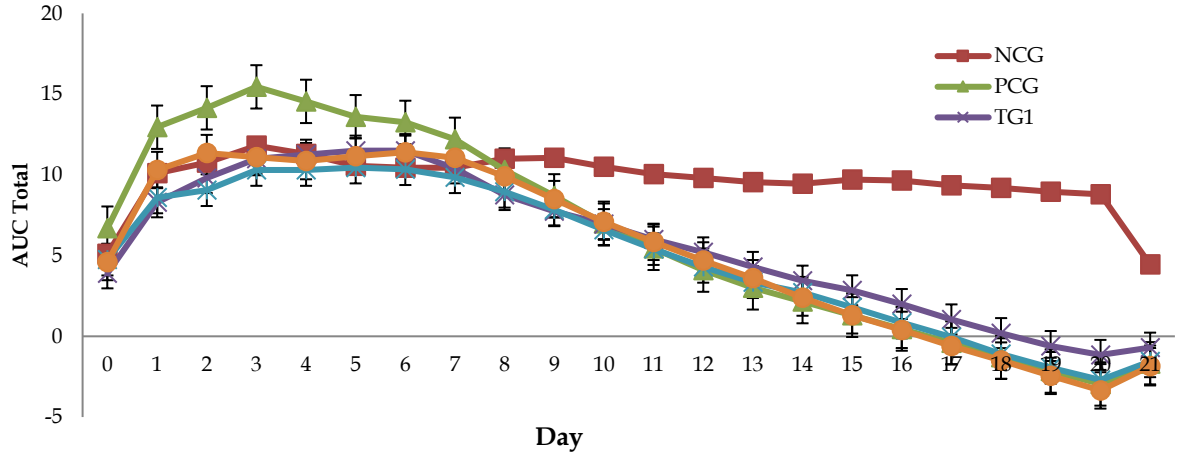

Figure 4. Total AUC value of joint thickness.

The AUC value describes the amount of active drug that reaches the systemic circulation[29]. The average AUC value was added from the 8th until the 21st day for each test animal. Then, the total average AUC value results were continued to calculate % inhibition. Percentage of inhibition to see how much inhibition was carried out by the compound given to the swelling that occurred—% inhibition for edema volume in **Table II** and % inhibition for joint thickness in **Table III**.

Table II. % inhibition of edema volume.

Treatment	AUC total ± SD	% inhibition
NCG	53.025 ± 0.3114	0
PCG	22.025 ± 1.6364	58.4629
TG1	38.200 ± 0.5239	27.9585
TG2	26.860 ± 1.6760	49.3446
TG3	24.750 ± 0.9364	53.3239

Table III. % inhibition of joint thickness.

Treatment	AUC total ± SD	% inhibition
NCG	131.500 ± 4.0498	0
PCG	33.950 ± 0.0822	74.1825
TG1	46.050 ± 0.4204	64.9809
TG2	34.450 ± 0.5952	73.8022
TG3	34.000 ± 0.1323	74.1444

Based on a previous research[30], the percentage decrease in edema volume for positive control methylprednisolone 15 mg/KgBW was 52.09%. Another study[21] stated that the % inhibition of edema volume for positive control of methylprednisolone 15 mg/KgBW was 75.7%, and joint thickness was 74.37%. So, the positive control has entered a good edema inhibition range to be compared with the treatment dose. The results of the % inhibition of edema volume and joint thickness show that doses of 100, 200, and 400 mg/KgBW can inhibit swelling, which was not much different from the positive control. Analysis data was performed to see if there were differences in each test group.

The results of statistical data for the edema volume and joint thickness for normality test results were sig >0.05 and the homogenous test sig <0.05. It means the data was normally distributed but not homogeneous, so it was continued with non-parametric tests using the Kruskal-Wallis test. The results of the Kruskal-Wallis test were sig <0.05, so there was a significant difference. We then continued with the Mann-Whitney test to see the treatment groups that have differences. The results of the Mann-Whitney test can be seen in **Tables IV** and **V**. Differences were found in the dose groups of 200 and 400 mg/KgBW against the positive group.

Table IV. Mann-Whitney test of edema volume.

	NCG	PCG	TG1	TG2	TG3
NCG	-	0.009*	0.009*	0.009*	0.009*
PCG	0.009*	-	0.009*	0.251	0.602
TG1	0.009*	0.009*	-	0.009*	0.009*
TG2	0.009*	0.251	0.009*	-	0.602
TG3	0.009*	0.602	0.009*	0.602	-

Note: *There was a significant difference

Table V. Mann-Whitney test of joint thickness.

	NCG	PCG	TG1	TG2	TG3
NCG	-	0.009*	0.009*	0.009*	0.009*
PCG	0.009*	-	0.009*	0.600	1.000
TG1	0.009*	0.009*	-	0.009*	0.009*
TG2	0.009*	0.600	0.009*	-	0.600
TG3	0.009*	1.000	0.009*	0.600	-

Note: *There was a significant difference

CONCLUSION

Ethanol extract of *E. bulbosa* bulbs can be used for anti-RA with effective doses of 200 and 400 mg/KgBW.

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AUTHORS' CONTRIBUTION

Conceptualization and methodology: **RM**, **HW**, **K**, **FS**. Simplicia preparation, extraction, and phytochemical screening: **RM**, **GRZ**. Anti rheumatoid arthritis test: **RM**, **HW**, **WBJ**, **GRZ**. Data analysis: **RM**, **HW**, **GRZ**, **K**, **FS**. Writing, review, and editing of manuscript: **RM**, **HW**, **WBJ**, **GRZ**.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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