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Original Article

IMMUNOACTIVITIES OF FRACTIONS OF *ELEPHANTOPUS SCABER* LINN LEAF IN NK CELLS, CD8$^+$ T CELLS, AND PERFORIN

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ABSTRACT

The goal of this study is to determine the effect of a fraction of *Elephantopus scaber* Linn leaf extract on NK cells, CD8$^+$ T cells, and perforin in male white mice using one dose of *Elephantopus scaber* Linn fraction at 30 mg/kg BW. In this study, 25 experimental animals were divided into 5 groups there are negative control, positive control, and 3 fractions of *Elephantopus scaber* Linn leaf, namely n-hexane, ethyl acetate, and butanol. Mice were treated with *Elephantopus scaber* Linn leaf fraction for 7 days after being previously stimulated by the SARS-Cov-2 virus antigen (inavac®). The animals were then analyzed on day 8. Duncan analysis was performed after One-Way ANOVA was used to evaluate the data. The test results showed that the concentration of NK cells increased slightly although it did not significantly increase (**p < 0.01; *p < 0.05) and the concentration of CD8$^+$ T cells and perforin significantly decreased (**p < 0.01; *p < 0.05). It is concluded that a fraction of *Elephantopus scaber* Linn leaf shows immunomodulatory activity.

KEYWORDS: *Elephantopus scaber* Linn; immunoactivities; fraction; NK cells; CD8$^+$ T cells; Perforin

1. Introduction

Medicinal plants are beneficial sources of a broad range of chemical compounds with varied structures and functions that demonstrate significant biological activity. The various biological activities of medical plants are antimicrobial, antiviral, anticancer, enzyme inhibitor and antioxidant, anti-inflammatory, anti-aging, anticoagulant effects, antihypertensive and neuroprotective ones [1]. According to recent statistics, the world contains 391,000 vascular plant species, and over 35,000 have the potential for therapeutic application. Furthermore, 25% of the bioactive components utilized in various medicines come from plants, and there are reports showing that at least 80% of the world's population rely on the medical plant [2]. There are some drugs that come from plants. The example is aspirin which was derived from willow bark and is used to relieve pain and prevent blood clots [3]. Morphine and codein come from the opium poppy (*Papaver somniverum*) [4]. Morphine is used to relieve severe pain and codein to relieve coughs and pain [5-6]. Caffeine is contained in coffee beans, tea leaves, cacao pods, kola nuts, and guarana and is used to relieve exhaustion and migraines [7]. Cocaine is derived from the coca plant and is used as an anesthetic and recreational drug [8]. Digitalis is derived from foxglove and used to cure arrhythmia [9].

Elephantopus scaber Linn, also known as Tapak Liman in Indonesia [10], is a medicinal plant native to tropical region around the world. It is a member of the Asteraceae family [14-15] and has long been utilized for medicinal purposes in numerous countries in Asia such as India, Vietnam, Malaysia, China and Indonesia [16]. The leaves, roots and herbs of *E. scaber* are widely used in traditional medicine [17-18]. *E. scaber* is used to treat wounds, gonorrhea, leukorrhea, mentrual complaints, cardiac problem, rheumatism, nephritis, hepatitis, edema, chest pain, fever, gout, scabies, scabs, dysentery, cough, fever, asthma, and bronchitis [12,16,19].

E. scaber contains a number of bioactive compounds that contribute to its therapeutic qualities. Previous studies reported that *E. scaber* contains phytochemicals, namely flavonoids, terpenoids, steroids, and saponins [20-22]. About 30 compounds have been identified in *E. scaber*, including 4 sesquiterpene lactones, 9 triterpenes, and 5 flavones [23]. Deoxyelephantopin (DET), isodeoxyelephantopin (IDET), scabertopin, and isoscabertopin are the main sesquiterpene lactones

derived from *E. scaber* which have anticancer activity [16,24-26]. This compounds can be isolated from the ethyl acetate fraction of *E. scaber* [27]. Based on research results, these sesquiterpene compounds are active as anticancer agents, among others breast [28-29], lung [30-32], tongue [33], and bladder cancer [34].

The immune system consists of the non-specific immune system and the specific immune system. The non-specific immune system is an innate immune system that responds to all foreign substances or antigen in the same way. The non-specific immune system is also called the innate immunity. Meanwhile, the specific immune system is an immune system that works specifically against antigens that enter cells or body fluids. This immune system is usually called the adaptive immunity [35]. NK (Natural Killer) cells, $CD8^+$ (Cluster of Differentiation) T cells and perforin have an importance role in the immune system. NK cells represent the innate immune system, $CD8^+$ T cells represents the adaptive immune system, while perforin is a molecule produced by NK cells and $CD8^+$ T cells. NK cells are cytotoxic lymphocytes that destroy target cells by secreting perforin and granzyme molecules. Cytotoxic T cells (CTL) are a critical subset of T cells that respond to intracellular infections like viruses and cancer cells. These cells have $CD8^+$ T cells molecules on their cell surfaces and can control infection by killing infected cells directly. They recognise peptide antigens given in the form of "MHC I:peptide complexes" by APCs. Antigen Presenting Cells (APCs): a group of immune cells that process and present antigens to T cells to trigger the adaptive cellular immune response. T lymphocytes initiate target cell death by recognizing the antigen with their T cell receptor (TCRs). CTL kill the target cell primarily through the release of cytotoxic mediators such as perforin and granzyme. Perforin creates pores in the membranes of diseased or malignant cells, which allow granzymes to enter and cause cell death [36]. Granzymes trigger apoptosis, or programmed cell death, in target cells by activating caspases, which are intracellular enzymes [37].

E. scaber leaf extract has immunostimulant activity [12,38,39], where the extract has the ability to increased T helper cells ($CD4^+$), cytotoxic T cells ($CD8^+$), prothymosit cells ($CD4^+$ $CD8^+$) and increased phagocytosis index, percentage of neutrophil, lymphocytes, monocyte, neutrophil, eosinophil and total leukocyte level [10,12,40]. However, research on the activity of new immunomodulators is limited to testing the whole *E. scaber* extract, and has not yet reached the level of fraction level.

2. Materials and methods

2.1. Materials

Materials that were utilized were fractions of *E. scaber* extract, aquadest, 0.5% NaCMC, n-hexane, ethyl acetate, butanol, physiological NaCl 0.9%, EDTA, COVID-19 vaccine (inavac®), white male mice, and the elisa kit was custom ordered from Bt lab: mouse NK cells ELISA kit (Bt Lab®), mouse $CD8^+$ T cells ELISA kit (Bt Lab®), and mouse perforin ELISA kit (Bt Lab®).

The instruments utilized were rotary evaporator (Buchi R-210®), beaker glass (Pyrex®), spatel, dark bottle, measuring cup (Pyrex®), desiccator, analytical balance (Mettler Toledo®), dropper, mask (Sensi®), gloves (Sensi®), micro pipette (Ecopipette®), animal cage, stemper mortal, probe needle (Terumo®), surgical instrument, centrifuge (Thermo Scientific®), microplate reader (Bio-Rad®), 48 plates wells.

2.2. Plant collection, identification and preparation

The leaves of *E. scaber* were collected in May 2023 from Desa Balai Kurai Taji, Pariaman, West Sumatra, Indonesia. The plant leaf was identified by a botanist at Herbarium ANDA Biology Department at Andalas University's Faculty of Mathematics and Natural Sciences in Indonesia, where a herbarium specimen was also deposited. The collected *E. scaber* leaves were separated from any attached dirt and washed with clean, running water. They were chopped and dried in a shady place for approximately 1 week. The dried leaves were weighed, ground into powder and sieved using a 60-mesh sieve.

2.3. Extraction of plant sample

The powdered plant material was extracted by maceration. Maceration is a simple extraction method that minimises the possibility of damaging the compounds in the sample through heating. The weighed sample was soaked in ethanol for about 72 hours with occasional stirring. After soaking, the sample was filtered using Whatman No. 1 filter paper. Subsequently a rotary evaporator was used to dry the filtered products at 45ºC and a pressure of 150 bar until a thick extract was obtained. The obtained thick extract was subsequently kept in a refrigerator at 4°C before further usage [41].

2.4. Fractionation of extract

Fractionation was carried out by dissolving the thick ethanol extract in a small volume of ethanol then diluting it with 500 ml of distilled water and stirring until all the extract dissolved. Next, the mixture was put into a separating funnel with a capacity of 2,000 ml and fractionated using 500 ml of n-hexane solvent or a ratio of 1:1. The separating funnel was shaken and fractionated until the n-hexane fraction was clear in color. After obtaining two different fractions, namely the n-hexane fraction and the water fraction, the water fraction was partitioned again using the solvent ethyl acetate in the same ratio, namely 500 ml or in a ratio of 1:1. Fractionation was carried out until the ethyl acetate fraction was clear in color. Next, the water fraction was partitioned again using 200 ml of butanol solvent until the butanol fraction was clear. Next, the n-hexane, ethyl acetate and butanol fractions were concentrated using a rotary evaporator until a thick fraction was obtained and the yield value was calculated.

2.5. Preparation of the animals for experiment

In this research, male white mice were used as experimental animals, which have passed an ethical review by the ethical commission team of the Faculty of Pharmacy, Andalas University with number 27/UN.16.10.D.KEPK-FF/2024.

Mice weighing 20-30 grams, as many as 25 male white mice, which had never received any medical treatment, were used. Previously, mice were acclimated to their surroundings for seven days before being employed as experimental animals. This allowed them to maintain consistent body weight, maintain good health, and control their diet by uniform food. These test subjects were split up into 5 groups, each of which had five mice

receiving a single fraction in one dosage. Mice were administered the inavac® vaccine on day 0 and the *E. scaber* leaf fraction for the next seven days. The mice's blood was extracted on the eighth day. After obtaining blood and serum, the ELISA method has been employed to measure the number of $CD8^+$ T cells and perforin [42]. The primary mechanism in the sandwich immunoassay ELISA method which is based on the creation of a bound between an antigen complex and 2 antibodies. The reason it is called a "sandwich" is that two layers of antibodies encase the antigens. The ELISA sandwich well has been coated with capture antibodies. This antibody will bind to the target antigen and then be held back by the antibody directly and indirectly, creating a sandwich-like structure [43,44].

2.6. Dosage determination

One dosage of 30 mg/kg BW of *E. scaber* leaf fraction was used in this research. On the eighth day, a neck artery guillotine was used to obtain blood. Serum was extracted from the blood by centrifuging it for 30 minutes at a speed of 3,000 rpm. Next, an ELISA technique was carried out on the serum to measure the amount of NK cells, $CD8^+$ T cells and perforin.

2.7. Analysis of data

Research data results were then examined statistically utilizing IBM SPSS software with the One-Way ANOVA along with subsequently the Duncan analysis.

Table 1. Extraction yield of extract and fraction of *E. scaber* leaf.

Weight of Sample (g)	Weight of Extract (g)	Yield (%)	Weight of Hexane Fraction (g)	Weight of Ethyl Acetate Fraction (g)	Weight of Butanol Fraction (g)
2,000	93.58	4.7	30.29	6.28	3.22

Table 2. Influence of fraction of *E. scaber* leaf on NK cells.

Groups	Influence of *E. scaber* fraction of leaf on NK cells (replication) (ng/ml)					Mean (ng/ml)	SD
	I	II	III	IV	V		
C-	20.28	21.79	15.79	19.01	16.51	18.68	2.52
C+	16.25	22.62	22.06	25.66	16.70	20.66	3.63
F1	22.14	23.94	20.66	21.67	22.09	22.09	1.06
F2	21.03	21.51	19.61	19.16	20.33	20.33	0.87
F3	22.71	20.16	22.51	21.35	21.46	21.64	0.92

Table 3. Influence of *E. scaber* Linn fraction of leaf on $CD8^+$ T cells.

Groups	Influence of *E.* scaber of leaf on CD8+ T cells (replication) (ng/ml)					Mean (ng/ml)	SD
	I	II	III	IV	V		
C-	21.94	20.91	19.89	22.58	21.31	21.33	0.92
C+	28.25	27.26	23.34	27.07	28.81	26.95	1.91
F1	17.41	17.73	16.70	17.18	17.02	17.21	0.35
F2	19.89	17.29	18.22	19.05	18.56	18.60	0.86
F3	18.33	14.23	17.08	15.64	17.41	16.54	1.44

Table 4. Influence of *E. scaber* fraction of leaf on perforin

Groups	Influence of *E. scaber* fraction of leaf on perforin (replication) (ng/ml)					Mean (ng/ml)	SD
	I	II	III	IV	V		
C-	28.86	26.12	24.49	28.48	22.77	26.14	2.32
C+	30.71	30.57	34.45	28.29	27.38	30.28	2.45
F1	19.72	21.30	21.78	20.49	20.76	20.81	0.70
F2	18.42	23.23	23.33	20.93	22.35	21.65	1.83
F3	18.65	18.29	19.22	18.93	18.76	18.77	0.30

Description:

C- : Control negative : not received induction and fraction

C+: Control positive : only received induction

F1 : Receive induction and hexane fraction 30 mg/kg body weight

F2 : Receive induction and ethyl acetate fraction dosage: 30 mg/kg body weight

F3 : Receive induction and butanol fraction dosage: 30 mg/kg body weight

3. Results and Discussion

Plants used as samples were listed and identified in ANDA herbarium, Biology Department, Mathematical and Natural Science Faculty, University of Andalas, West Sumatra with an identification number 646/K-ID/ANDA/X/2023. Identification showed that the plant sample was *E. scaber* Linn.

The process for producing *E. scaber* leaf extract starts with gathering fresh *E. scaber* leaves, washing them under running water to remove impurities, and letting them air dry to produce dry simplicia. Next, the dried simplicia is ground up in a blender to create powder, and a maceration sample is made using 70% ethanol solvent. From 10,000 grams of fresh *E. scaber* Linn leaf, 2,000 grams of dried simplicia were obtained. A thick extract weighing 93.58 grams was obtained from 2,000 grams of simplicia powder, with a yield value of 4.7%. The thick extract was then fractionated using 3 solvents based on their polarity level, namely hexane, ethyl acetate and butanol. The fractionation results obtained were: a hexane fraction weighing 30.29 grams; ethyl acetate fraction 6.28 grams; and butanol fraction 3.22 grams.

3.1. Assay of NK Cells Concentration

NK cells are the main component of the innate immune system, which is the body's first line of defense against threats. NK cells are a type of lymphocyte that identify and destroy infected cells. NK cells can mediate their cytotoxic activity via 2 distinct pathways. They can release cytotoxic granules containing perforin and granzymes, or they can induce death receptor-mediated apoptosis by expressing TRAIL and/or Fas ligand (FasL) to engage TRAIL-R1/-R2 or CD95/Fas, respectively, on the surface of diseased cells [45]. NK cells also work through the release of cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) which are involved in innate or adaptive immune responses [46]. NK cells begin to appear on the second day after infection. NK cells identify the viral-encoded protein m157 on infected cells by activating the Ly49H receptor, prompting cytotoxicity and cytokine release that can regulate subsequent immune responses [47]. NK cells, apart from being the first line of fighting infected cells, are also the first line in the spread of diseased cells such as cancer cells [48].

According to the ELISA test results (Fig. 1), NK cells count was the highest in the hexane fraction group and it was 22.09 ng/ml. That is the group of mice that received NaCMC suspension and SARS-Cov-2 virus antigen (inavac®) then given *E. scaber* Linn hexane fraction with dose 30 mg/kg BW. The statistical test of One-Way ANOVA followed with post-hoc Duncan analysis revealed that there were no significant differences (**p < 0.01; *p < 0.05) among five treatment groups. The mean quantity of natural killer cells from male white mice administered *E. scaber* Linn leaf fraction and SARS-Cov-2 virus antigen, ranging from group C-, C+, F1, F2 to F3, was determined to be 18.68; 20.66; 22.09; 20.33 and 21.64 ng/ml, respectively.

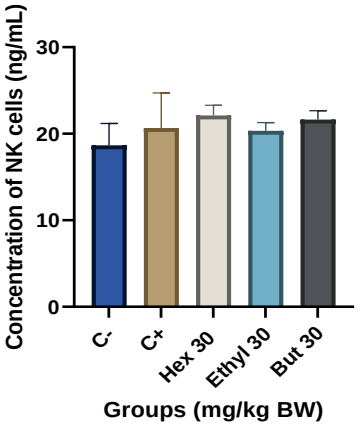

Fig. 1. Influence of *E. scaber* fraction of leaf on NK cells. The values shown are the mean ± standard deviation (n=2). The variance among the control and treatment groups was assessed for statistically significant utilizing one-way ANOVA, then analyzed using post-hoc Duncan analysis. Asterisks (*) represent significant differences with control+ values (**p < 0.01;*p < 0.05).

3.2. Assay of CD8⁺ T Cells Concentration

CD8⁺ T cells are a specific immune system that can kill infected cells and also inhibit the growth of cancer cells. Similar to NK cells, CD8⁺ T cells also work by releasing cytolytic molecules and proinflammatory cytokines [49,50]. CD8⁺ T cells will appear around day 5 to day 8 of infection [51]. After encountering an antigen, naïve CD8⁺ T cells differentiate into effector cells capable of infiltrating cells or tissues and eliminating malignant or pathogen-infected cells that express the appropriate peptide-MHC-I combination. Once the antigen is eliminated, the effector population contracts, and the cells that remain develop into central memory (CM) and long-lived effector memory (EM) cells [49].

According to the ELISA test results shown in Fig. 2, among 3 fractions CD8⁺ T cells count was the highest in the ethyl acetate fraction group and it was 18.60 ng/ml. That is the group of mice which received NaCMC suspension and SARS-Cov-2 virus antigen (inavac®) then were given *E. scaber* Linn ethyl acetate fraction with dose 30 mg/kg BW. Based on data, the control experimental animals have higher CD8⁺ T cells concentration than the experimental animals that received the fraction. This may be due to the relatively short observation time of only 7 days, whereas the CD8⁺ T cells start appearing around days 5 to 8 after infection begins. The statistical test of One-Way ANOVA followed with post-hoc Duncan analysis revealed that there are significant differences (**p < 0.01; *p < 0.05) among five treatment groups. The mean quantity of CD8⁺ T cells from male white mice administered *E. scaber* Linn leaf fraction and SARS-Cov-2 virus antigen, ranging from group C-, C+, F1, F2 to F3, was determined to be 21.33; 26.95; 17.21; 18.60 and 16.54 ng/ml, respectively.

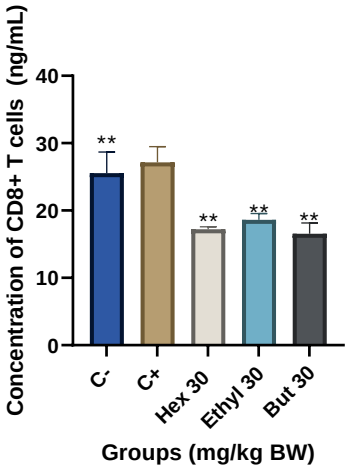

Fig. 2. Influence of *E. scaber* fraction of leaf on $CD8^+$ T cells. The values shown are the mean ± standard deviation (n=1). The variance among the control and treatment groups was assessed for statistically significant utilizing one-way ANOVA, then analyzed using post-hoc Duncan analysis. Asterisks (*) represent significant differences with control+ values (**p < 0.01;*p < 0.05).

3.3. Assay of Perforin Concentration

Perforin is a glycoprotein compound released by NK cells and $CD8^+$ T cells which plays a role in the immune system [52,54]. Perforin produced from cytolytic granule interacts with target cell membrane and generates a pore leading to activation of cell cytotoxicity [52]. Perforin is a final component in a complex chemical signaling cascade that leads a targeted cell to rapidly induce apoptosis, a sort of programmed cell death [55].

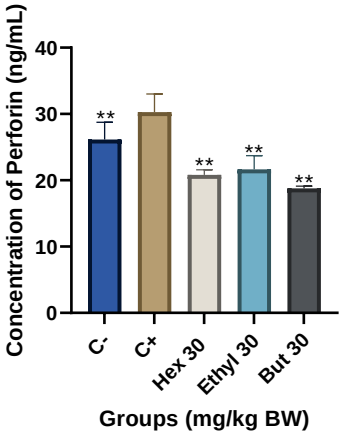

Fig. 3. Influence of *E. scaber* fraction of leaf on perforin concentration. The values shown are the mean ± standard deviation (n=2). The variance among the control and treatment groups was assessed for statistically significant utilizing one-way ANOVA, then analyzed using post-hoc Duncan analysis. Asterisks (*) represent significant differences with control+ values (**p < 0.01;*p < 0.05).

According to the ELISA test results shown in Fig. 3, among 3 fractions perforin concentration was the highest in the ethyl acetate fraction group and was 21.64 ng/ml. That is the group of mice that received NaCMC suspension and SARS-Cov-2 virus antigen (inavac®) then were given *E. scaber* ethyl acetate fraction with dose 30 mg/kg BW. The statistical test of One-Way ANOVA followed with post-hoc Duncan analysis revealed that there are significant differences (**p < 0.01; *p < 0.05) among five treatment groups. The mean quantity of perforin concentration from male white mice administered *E. scaber* leaf fraction and SARS-Cov-2 virus antigen, ranging from group C-, C+, F1, F2 to F3, was determined to be 26.14; 30.28; 20.81; 21.65 and 18.77 ng/ml, respectively.

4. Conclusion

The findings of this investigation demonstrate the potential immunomodulatory activity of three *E. scaber* fractions: hexane, ethyl acetate, and butanol. These findings support previous research on the immunomodulatory action of this plant extract. The test results showed that the concentration of NK cells from groups C-, C+, F1, F2, and F3 was determined to be 18.68; 20.66; 22.09; 20.33 and 21.64 ng/ml, $CD8^+$ T cell concentration 21.33; 26.95; 17.21; 18.60 and 16.54 ng/ml, and perforin 26.14; 30.28; 20.81; 21.65 and 18.77 ng/ml. The data were statistically evaluated using IBM SPSS software using One-Way ANOVA, followed by Duncan analysis. The test results revealed that the concentration of NK cells increased slightly although it did not significantly increase (**p < 0.01; *p < 0.05), while the concentration of $CD8^+$ T cells and perforin significantly decreased (**p < 0.01; *p < 0.05). It is concluded that fractions of the *E. scaber* leaf exhibit immunomodulatory function. More research is needed to determine the immunomodulatory activity of *E. scaber* and its relationship to phytochemical content, so the future research on this plant about immunomodulator activities can produce immunomodulator products that are safe and have few side effects.

Author Contributions: Mega Yulia: Investigation, Formal analysis, Writing original draft. Yufri Aldi: Conceptualization, Validation, Supervision. Hansen Nasif: Validation, Data curation. Fatma Sri Wahyuni: Validation, Supervision. Dachriyanus: Conceptualization, Project administration, Funding acquisition.

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Conflict of Interest: There are no conflicts of concern declared by authors.

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