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Original Research Article

Immunostimulant Studies of NK Cells, CD8$^+$ T Cells and Perforin From Ethanol Extract of *Elephantopus scaber* Linn. Leaf

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ABSTRACT

Elephantopus scaber Linn , traditionally, boosts the immune system, highlighting its potential for scientific investigation. This study aims to determine the effect of *E. scaber* leaf extract on NK cells, CD8$^+$ T cells and perforin in male white mice using three different doses (10, 30, and 100 mg/kg bw). A total of 25 experimental animals were divided into five groups and treated with *E. scaber* leaf extract for seven days after being stimulated by the SARS-Cov-2 virus antigen (inavac®). On day eight, the animals were then analyzed. The results of NK cells evaluation from negative control, positive control, and extract with concentrations of 10, 30, and 100 mg/kg bw were 18.68, 20.66, 22.08, 21.14, and 21.87 ng/ml. The results of CD8$^+$ T cells were 21.33, 26.95, 24.53, 33.85, and 24.87 ng/ml. Meanwhile, the perforin concentrations were 26.14, 30.28, 24.27, 33.09, and 26.38 ng/ml. Duncan's analysis was performed after a one-way ANOVA to evaluate the data. At an extract dose of 30 mg/kg bw, the test results showed that the concentration of CD8$^+$ T cells significantly increased (**$p < 0.01$; *$p < 0.05$), and the concentration of perforin increased, although insignificantly. On the other hand, the concentration of NK cells increased most at a dose of 10 mg/kg bw. These findings conclude that the leaf extract of *E. scaber* showed immunostimulant activity.

Keywords: *Elephantopus scaber*, Immunostimulant, Natural Killer cells, Cytotoxic T cells, Perforin

Introduction

Coronavirus disease 2019 (COVID-19), also referred to as 2019 new coronavirus (2019-nCoV) is a highly transmissible respiratory disease.[1,2] Since its emergence, COVID-19 has occurred and mutated with various new variants of SARS-CoV-2.[3] It is often perceived as the largest pandemic experienced by humans,[4] and has led to an enormous number of illnesses and mortality. These infections often manifest in a unique type of pneumonia, typically causing severe symptoms of acute respiratory syndrome. Naturally, the body can protect itself from such infection via the immune system. According to Jain *et al*.,[5] there are two categories of immune systems: innate immunity and adaptive immunity. Innate immunity, also known as nonspecific immunity, is the first protection against microbes that attack the body, for example, macrophages, neutrophils, monocytes, basophils, mast cells, dendritic cells (DC), natural killer (NK) cells, and nonimmune cells like epithelium. Meanwhile, adaptive immunity, also known as specific immunity, is the second protection, which works as a more targeted defense by responding to specific antigens, a process mediated by B and T lymphocytes.[6] Although slower to activate, adaptive immunity is crucial in long-term protection.

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The immune system can be improved with certain compounds called immunostimulants. These substances can boost immune system function by activating adaptive and innate immunity.[7] They can serve as a therapeutic option for treating COVID-19.[8] Recent research based on *in vitro* and *in vivo* data demonstrated that some medicinal herbs can be used as natural immunostimulants to boost the immune system in preventing and treating COVID-19 infection.[9–14] However, current sources of natural immunostimulants are still limited to *Phylantus niruri* and *Echinacea purpurea*. Phylantus niruri or meniran has become the only immunostimulant classified as a phytopharmaceutical in Indonesia.

Given the limited availability of immunostimulants from natural ingredients and based on Regulation No HK.02.02/IV.2243/2020 of the Republic of Indonesian Ministry of Health on the Use of Traditional Medicines for Disease Prevention, Health Maintenance and Medical Care, including during the health emergency or COVID-19 disaster, it is necessary to explore other plant-based alternatives derived with relatively lower toxicity and fewer side effects compared to synthetic immunostimulants.[15–19] One promising alternative is *E. scaber*, a plant commonly found in Europe, Asia, Africa, Australia, and Indonesia. This plant belongs to the Asteraceae family and has been traditionally used for various treatments such as treating fever, gout, dysentery, hepatitis, infections, wound healing, nephritis, edema, chest pain, scabies, cough, tonics, and respiratory diseases, such as bronchitis and asthma. It has also been reported effective in treating diuretics, dysuria, diarrhea, arthralgia, rheumatic arthritis, antifebrile, leukemia, and cancer.[15–19] The chemical contents of *E. scaber* include sesquiterpenes, triterpenoids, steroids, flavonoids, tannins, saponins, and essential oils.[20–24] These compounds are associated with several pharmacological activities. For instance, sesquiterpene lactone compounds isolated from whole *E. scaber* plants demonstrated effective anti-inflammatory activity on LPS-stimulated RAW 264.7 cells with IC_{50} concentrations ranging from 6.27 ± 0.18 to 18.31 ± 1.38 μM.[25] The ethanol extract of *E. scaber* leaves has demonstrated immune-boosting potentials in mice with 10, 30, and 100 mg/kg bw doses.[26] Moreover, *E. scaber* leaves have been known to have antibacterial activity with diameter of zone

inhibition against *Staphylococcus aureus* 20 mm at the concentration of 100 mg/ml.[27] Similarly, Efendi *et al*.,[28] added that *E. scaber* exhibits antibacterial activity against various pathogenic bacteria, particularly in ethyl acetate fractions, with MIC values of 19-156 µg/mL against *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC 25175, *Vibrio cholerae* Inaba, and *Pseudomonas aeruginosa* ATCC 27853. These findings show the plant's potential as a sustainable source of antibacterial agents.

Additionally, several studies have found that sesquiterpene lactone compounds from *E. scaber* show remarkable antitumor activity on a variety of cancer cell types, including the most common type of brain tumor, known as glioma, which is malignant and has a high mortality rate.[29] Despite the promising findings, no studies have specifically investigated the immunostimulant activity from *E. scaber* that are meant to prevent or treat COVID-19. This gap in research has prompted scientific interest in exploring how *E. scaber* leaf extract may combat SARS-CoV-2 infection by looking at its effects on immune responses involving NK cells, $CD8^+$ T cells and perforin.

Materials and Methods

Materials

The materials used in this study included *E. scaber* extract, aquadest, 0.5% NaCMC, 70% ethanol, ethanol P, hexane, ethyl acetate, methanol, 0.1% formic acid, 0.9% physiological NaCl, Mayer's reagent, Dragendorf reagent, Mg metal, HCl, $FeCl_3$, anhydrous acetic acid, and concentrated sulphuric acid were purchased from Bratachem company, Indonesia. F_{254} silica gel TLC plate and EDTA were obtained from Merck, Germany, mouse NK cells ELISA kit (Bt Lab®), mouse $CD8^+$ T cells ELISA kit (Bt Lab®), mouse perforin ELISA kit (Bt Lab®), and COVID-19 vaccine (inavac®) were also used. As biological materials, the study used white male mice ddy strain. The instruments included a rotary evaporator (Buchi R-210® from Sigma and Aldrich, USA), a Shimadzu liquid chromatography system (Shimadzu LC 20 AD® from Shimadzu, Japan), oven (Carbolite Gero®, German), water bath (WTB 15 with Ring from Memmert®, German), analytical balance (LA84E from Mettler Toledo®, USA), micropipette (Ecopipette®), centrifuge (Pico™ 21 Microcentrifuge from Thermo Scientific®, USA), microplate reader (xMark™ from Bio-Rad®, USA).

Plant Collection, Identification and Preparation

The leaves of *E. scaber* were collected from Balai Kurai Taji Village, Pariaman, West Sumatera, Indonesia (-0.6336178761446255, 100.16273166388605). 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. After collection, the leaves were separated from any attached dirt and washed under clean, running water. They were then chopped and dried in a shady place for approximately one week. Once dried, the leaves were weighed, ground into powder, and sieved using a 60-mesh sieve.

Extraction of Plant Sample

E. scaber powder material of 2,000 gram was extracted through maceration method using 5 liters of ethanol. The maceration method was chosen because this method can avoid damage to compounds due to heating. The powdered sample was carefully weighed and soaked in ethanol for 72 hours. After soaking, the mixture was filtered using Whatman No. 1 filter paper. Subsequently, a rotary evaporator was used to dry the filtered products at 45ºC to produce the thick extract. The extract was subsequently kept in a refrigerator at 4°C until further use.

Organoleptic test

Organoleptic tests were conducted using the five senses to describe the characteristics of *E. scaber* extract, including its shape (solid, dry powder, thick, liquid), color, odor (aromatic, odorless, etc.) and taste. The percentage yield of extract produced (%) was calculated by comparing the weight of the obtained *E. scaber* extract to the weight of the raw material.[30]

$$\text{Yield (\%)} = \frac{\text{weight of } E.\ scaber \text{ extract}}{\text{weight of raw materials}} \times 100\%$$

Phytochemicals screening

The initial qualitative assessment of the phytochemicals of *E. scaber* extract was carried out by chemical reactions to identify the main classes of compounds (flavonoids, alkaloids, saponins, phenolics, steroids and terpenoids) present in extract following standard method.[31]

Qualitative HPLC analysis

Analysis of extracts and standards were performed by Liquid chromatography using a Shimadzu HPLC apparatus (Shimadzu LC 20 AD). An accurately weighed standard solution was prepared by dissolving the reference standards in water: acetonitrile (66:34, v/v) to a final concentration of 2.50 µg mL-1 for deoxyelephantopin. For *E. scaber* extract, 100 mg *E. scaber* extract leaves was transferred into a 50 ml volumetric flask adjusted with water : acetonitrile (66: 34, v/v) and sonicated for 15 min, filtered through a 0.45 µm membrane.

Qualitative HPLC analysis

The experiment was performed using a solvent system consisting of a mobile phase methanol : 0.1% formic acid (98 : 2) v/v; stationary phase C_{18}; column temperature 26ºC; injection volume 20 µl, VWD detector λ 210 nm; flow rate 0.4 ml/min and 0.5 ml/min.

Animal Preparation

In this study, male white mice were used as experimental animals. The ethical use of this animal was approved by the ethical commission team of the Faculty of Pharmacy, Andalas University, under approval number 27/UN.16.10.D.KEPK-FF/2024. The experiment involved 25 mice weighing 20-30 grams that had never received any medical treatment. Before the experiment, mice were acclimated to their surroundings for seven days before being employed as experimental animals. This allowed them to maintain consistent body weight, good health, and a uniform diet.

Following that, the test subjects were divided into five groups, with each group consisting of five mice. Each group received a single extract in 3 distinct dosage ranges. On day 0, mice were administered the inavac® vaccine and the *E. scaber* leaf extract for seven days. The mice's blood was extracted on the eighth day. After obtaining blood and serum, the ELISA method was employed to measure the number of NK cells, $CD8^+$ T cells and perforin. The primary mechanism of the sandwich immunoassay, the ELISA method, involves connecting an antigen complex with two antibodies. It is called a "sandwich" because it has two layers of antibodies encasing the antigens. The ELISA sandwich well was coated with capture antibodies. This antibody would bind to the target antigen and then be held back by the antibody directly and indirectly, creating a sandwich-like structure.[32,33]

Dosage Determination

Three different dosages (10, 30, and 100 mg/kg bw) of *E. scaber* leaf extract were 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 3000 rpm. Next, an ELISA technique was carried out on the serum to measure the amount of NK cells, $CD8^+$ T cells and perforin.

Data Analysis

The research results were then examined statistically using the One-Way ANOVA and the Duncan analysis. All these statistical calculations were facilitated by the IBM SPSS software (SPSS Inc®) 27 release 2020. Tests were performed in repetition five times and the results were presented as mean ± standard deviation (SD). Differences in p values of <0.01 and <0.05 were considered significant.

Results and Discussion

From 2 kg of dried *E. scaber* leaves, 98.58 g of extract was obtained with a yield of 4.7%. The identification results at the ANDA herbarium showed that the plant sample was *Elephantopus scaber* Linn. As part of the proximate analysis of the sample, it was standardized to give a drying shrinkage of *E. scaber* leaf extract of 7.66%. This value complies with Indonesian Herbal Pharmacopoeia's requirement, which specifies

that the drying shrinkage of leaf extract should not exceed 10%.[34] Additionally, this study generated a thick extract of *E. scaber* leaf with a total ash percentage of 2.53%, which aligns with the requirement of Indonesian Herbal Pharmacopoeia, stating that the overall quantity of ash contained in a leaf extract should not exceed 10%.[34] Sensory evaluation or organoleptic tests were also carried out on *E. scaber* extract, which aims to identify the leaf extract characteristics. The assessment can be done through the five senses. The results revealed a dark green color, thick consistency, pungent odor, and a bitter taste. In terms of phytochemical or secondary metabolite content, these findings indicate that phenolics, flavonoids, terpenoids, steroids, and saponins are present in *E. scaber* leaf extract (Table 1).

Table 1: Phytochemical screening of *E. scaber* leaf

Phytoconstituens	**Result**
Flavonoids	+
Alkaloids	-
Saponins	+
Phenolics	+
Steroids	+
Terpenoids	+

Description : (+) = contain, (-) = not contain

Analysis using TLC involves separating chemical components based on the principles of adsorption and partition determined by the stationary phase (adsorbent) and the eluent. Chemical components move up with the mobile phase due to the absorption capacity of the adsorbent and are separated based on their polarity.[35] In this research, TLC analysis of the *E. scaber* extract was conducted by spotting it on the TLC plate with the mobile phase hexane: ethyl acetate in a ratio of 90:10 v/v. The results visible under 365 nm UV light show three spots with Rf values of stains 1, 2, and 3, respectively, which were 0.19, 0.27, and 0.51. Fluorescence under UV light 365 nm indicates that the compound has a conjugated double bond, also called a chromophore, and has an auxochrome group in its structure (Figure 1).

Figure 1: Thin Layer Chromatography (TLC) profile of *E. scaber* extract using silica gel 60 F_{254} as stationary phase and hexane:ethyl acetate 90:10 v/v as development solvent; observed under uv 365 nm

According to the ELISA test results, the NK cell count was highest in group D1, measuring 22.08 ng/mL. This group received *E. scaber* leaf extract at 10 mg/kg bw from days 1 to 7. ANOVA statistical analysis, followed by the post-hoc Duncan test, revealed no significant differences among the five treatment groups (**$p < 0.01$; *$p < 0.05$). The means NK cells in male white mice administered varying doses of *E. scaber* leaf extract and SARS-Cov-2 virus antigen, ranging from group C-, C+, D1, D2, and D3, were determined to be 18.68; 20.66; 22.08; 21.14 and 21.87 ng/mL (Table 2, Figure 2).

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

Groups	Influence of *E. scaber* extract of leaf on NK cells (replication) (ng/ml)					Mean (ng/ml)	SD
	I	II	III	IV	V		
C-	20.28	21.79	15.80	19.00	16.51	18.68	2.25
C+	16.25	22.62	22.06	25.66	16.70	20.66	3.63
D1	22.83	22.76	20.76	24.02	20.01	22.08	1.47
D2	21.51	19.81	21.79	21.79	20.83	21.14	0.75
D3	18.94	22.48	22.20	25.44	20.28	21.87	2.21

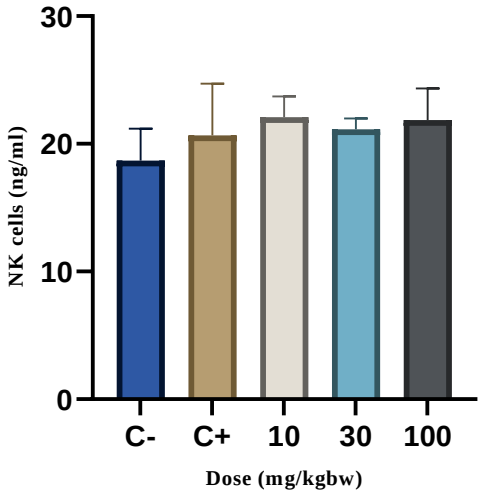

Figure 2: Influence of *E. scaber* extract of leaf on NK cells

NK cells are necessary for innate protection from infections inside cells, which shows cytolytic spontaneous action against stressed cells, including tumors or neoplasms, and virus-infected cells.[36,37] Although NK cells are equipped to combat pathogens, they must first undergo an initial activation phase to function fully. This phase involves the action of multiple cytokines, such as type I interferons and the activating cytokines IL-2, IL-12, IL-15, and IL-18, which can be made by activated antigen-presenting cells or virally infected cells.[38,39] In cells that are not affected, interferon also increases their resistance to viruses. NK cells are proteins that have the power to destroy cells contaminated by a virus by using CD16-mediated antibody-dependent cellular cytotoxicity (ADCC).[39] Numerous cytokines, in combination or separately, increase NK cell survival, proliferation, cytotoxicity, and cytokine production, such as interferon-γ (IFN-γ) production. These processes enable NK cells to have special abilities that allow them to detect viral infections and react swiftly.[40,41] Hence, they frequently cooperate with other immune system responses to facilitate the development of antiviral immunity.[39]

ELISA test results showed that the $CD8^+$ T cell count was highest in group D2, measuring 33.85 ng/mL. This group received the *E. scaber* leaf extract at 30 mg/kg bw from days 1 to 7. ANOVA analysis, followed by the post-hoc Duncan test, revealed significant differences among the five treatment groups (**$p < 0.01$; *$p < 0.05$). The means

$CD8^+$ T cells in male white mice administered varying doses of *E. scaber* leaf extract and SARS-Cov-2 virus antigen, ranging from group C-, C+, D1, D2, and D3, were determined to be 21.33; 26.95; 24.53; 33.85 and 24.87 ng/ml (Table 3, Figure 3).

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

Groups	Influence of *E. scaber* extract 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
D1	24.89	24.83	24.83	23.40	24.71	24.53	0.57
D2	34.16	34.42	32.58	31.99	36.09	33.85	1.45
D3	23.82	27.44	26.22	22.35	24.53	24.87	1.79

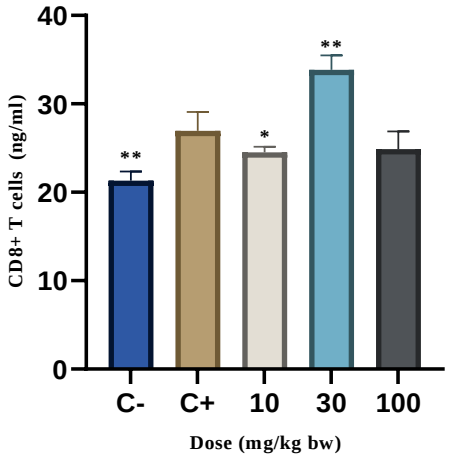

Figure 3: Influence of *E. scaber* extract of leaf on $CD8^+$ T cells

$CD8^+$ T cells play a crucial role in controlling infections and conducting cancer immune surveillance, forming an integral part of the adaptive (acquired) immune system.[42,43] Major Histocompatibility Complex Class I (MHC-I) molecules on antigen-presenting cells (APCs) display foreign peptides that are recognized through the interaction of both the T cell receptor (TCR) and the CD8 co-receptor.[44] Cytokines like IL-2, IL-6, IL-12, and IL-23 even strengthened this process. Cross-reactive and naïve memory T cells become active, proliferate, and mature. Once activated, they left the lymphoid tissue and entered the infection site.[45] After contact with an antigen, naïve $CD8^+$ T cells undergo differentiation to become effector cells that can infiltrate cells or tissues and eliminate malignant or pathogen-infected cells that express the appropriate peptide-MHC-I combination. After eliminating the antigens, the population of effectors contracts, leaving behind cells that develop into central memory (CM) and long-lived effector memory (EM) cells.[46]

The results also revealed that the perforin content was highest in group D2, measured at 33.09 ng/mL, with the extract dose of 30 mg/kg bw from days 1 to 7. Similarly, the statistical test of One-Way ANOVA, followed by the post-hoc Duncan test, revealed significant differences among the five treatment groups (**$p < 0.01$; *$p < 0.05$). The mean quantity of perforin from male white mice administered varying doses of *E. scaber* leaf extract and SARS-Cov-2 virus antigen, ranging from group C-, C+, D1, D2, and D3, was determined to be 26.14; 30.28; 24.27; 33.09 and 26.38 ng/ml (Table 4, Figure 4).

Table 4: Influence of *E. scaber* extract of leaf on perforin

Groups	Influence of *E. scaber* extract of leaf on perforin (replication) (ng/ml)					Mean (ng/ml)	SD
	I	II	III	IV	V		
C-	28.86	26.11	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
D1	24.99	26.69	25.03	19.61	25.03	24.27	2.42
D2	33.93	32.42	33.46	34.69	30.95	33.09	1.29
D3	27.23	29.62	26.69	26.26	22.09	26.38	2.44

Description:
C- : Control negative : did not received induction and extract
C+ : Control positive : only received induction
D1 : Received induction and extract dosage 10 mg/kg bw
D2 : Received induction and extract dosage 30 mg/kg bw
D3 : Received induction and extract dosage 100 mg/kg bw

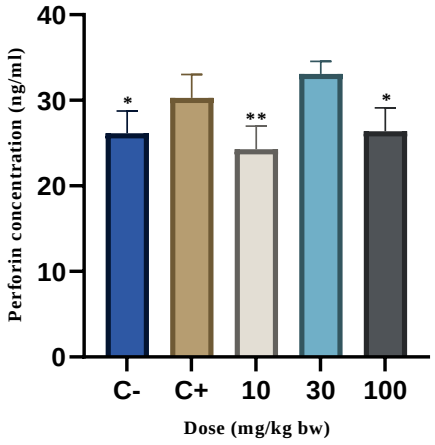

Figure 4: Influence of *E. scaber* extract of leaf on perforin

The glycoprotein perforin is responsible for creating pores in target cell membranes.[47,48] It polymerizes to make these pores in the membrane of target cells, with NK cells and $CD8^+$ T cells serving as the primary sources of perforin.[47] Perforin is a key mediator of tissue damage caused by "killer lymphocytes", such as $CD8^+$ cytotoxic T lymphocytes (CTLs) and NK cells. Through a contact-dependent process, both cell types can eliminate target cells that the immune system deems dangerous. Regarding CTLs, clonotypic receptors on the cell surface identify a foreign or mutant peptide antigen taken from inside the cell and displayed major histocompatibility complex (MHC) proteins on the target cell surface. If the binding is strong enough, a stable

immunological synapse is formed with the target cells due to receptor clustering, signaling, and remodeling of the actin cytoskeleton on the CTL. Then, after being recruited and traveling through the microtubular structure to the cell-cell interaction site, highly specialized and prepared cytotoxic secretory vesicles (CSVs) inside the CTL cytoplasm release a cocktail of cytotoxins to the immunological synapse via exocytosis. As previously stated, perforin acts as the final component in a complex chemical signaling cascade that rapidly leads a targeted cell to induce apoptosis, a programmed cell death.[49]

The study results show that the ethanol extract of *E. scaber* leaf has immunostimulant activity. This may be due to some secondary plant metabolites, e.g., deoxyelephantopin, which has demonstrated potential pharmacological activity. The HPLC results show a deoxyelephantopin compound in the *E. scaber* ethanol extract (Figure 5). *E. scaber* extract (A), flow rate 0.4 mL/min, compared with standard deoxyelephantopin flow rate 0.4 mL/min (B), has almost the same retention time, 3.908 min (A) and 3.971 min (B). *E. scaber* extract (C) flow rate 0.5 mL/min compared with standard deoxyelephantopin flow rate 0.5 mL/min (D) has almost the same retention time, 3.141 min (A) and 3.176 min (B).

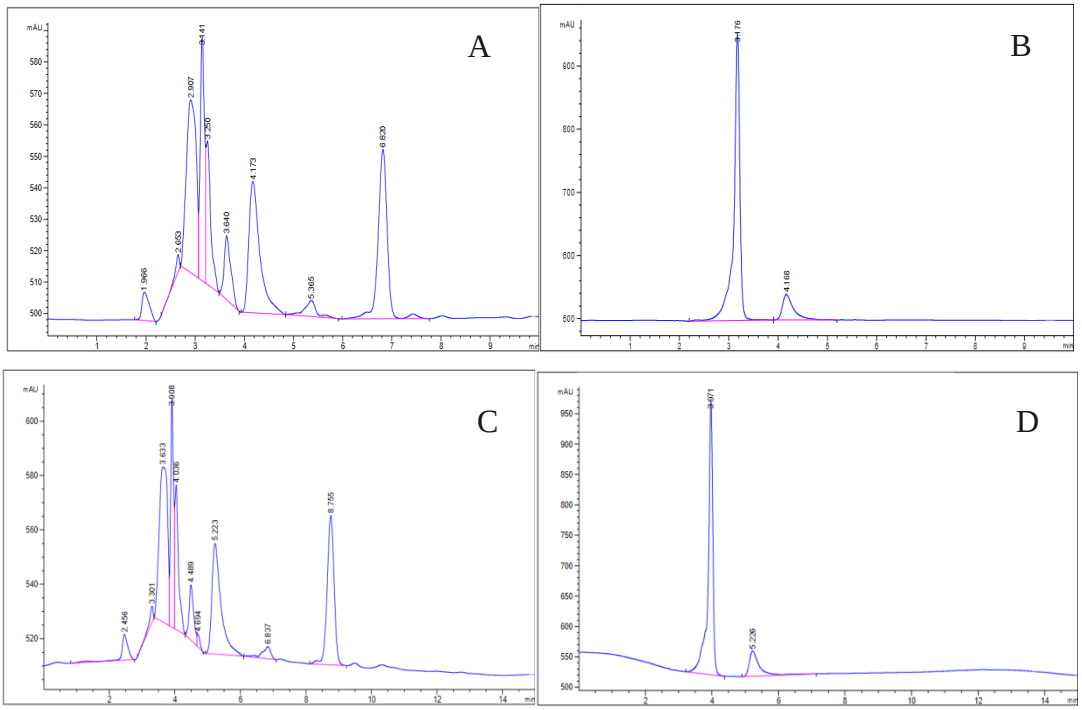

Figure 5: HPLC profiles with mobile phase methanol : 0.1% formic acid (98 : 2) v/v; stationary phase C_{18}; column temperature 26°C; injection volume 20 µl, VWD detector λ 210 nm. (A) *E. scaber* leaf extract flow rate 0.4 ml/min, (B) Deoxyelephantopin compound flow rate 0.4 ml/min, (C) *E. scaber* leaf extract 0.5 ml/min, (D) Deoxyelephantopin compound flow rate 0.5 ml/min.

Conclusion

This study showed immunostimulant activity of *E. scaber* leaf. This findings revealed a significant increase in the population of $CD8^+$ T cells and NK cells which are key components in cellular immunity. Furthermore, the result of this study showed an increase in the expression of the perforin protein which is an indicator of the cytotoxic activity of immune cells. The increase in specific immune cell population and perforin expression collectively indicates that the *E. scaber* leaf extract is able to modulate and enhance cellular immune responses. The data obtained provide a scientific basis for the development of phytopharmaceuticals derived from *E. scaber* leaf, with special potential as immunostimulant agents. Furthermore, in-depth studies on the active compounds from *E. scaber* leaf and their mechanism of action in improving the immune system in NK cells, $CD8^+$ T cells, and perforin is needed to fully utilize its therapeutic potential.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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