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Original Research Article

Activities of *Hydrocotyle sibthorpioides* Lam. Extract in Capsule on Natural Killer and CD8 Cells in Human

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ABSTRACT

Hydrocotyle sibthorpioides Lam. has been used by Indonesians to increase immunity. The present study was therefore conducted to investigate the effect of *Hydrocotile sibthorpioides* Lam. extract in capsules on the activity of natural killer (NK) and CD8 cells in humans during the first phase of clinical trials. Twenty participants were recruited for the study and divided into two groups. The first group received nothing except an additional ingredient as a placebo. Also, the second group was administered a preparation test at a dose of 67 mg per person. Blood was obtained before and after the test preparation was administered, which was done once daily for three days. The protein concentrations of NK and CD8 cells were measured by the enzyme-linked immunosorbent assay (ELISA) kit. The results showed that the average activity of NK cells before giving the preparations to the first group was 81.644 U/mL, and after administration was 81.910 U/mL. In contrast, the average activity of NK cells of the second group was 81.998 and 83.541 U/mL, respectively. The concentrations of CD8 cells in the first group were 230.160 and 235.575 U/mL before and after administration, respectively. Compared to the second group, the concentrations of CD8 cells were 238.407 and 335.323 U/mL, respectively. The findings of this study indicate that the *H. sibthorpioides* extract could increase the NK and CD8 cellular activities, which may improve the body's capacity to destroy virus invaders.

***Keywords*:** CD8 cell, Clinical trial, *Hydrocotyle sibthorpioides* Lam., Immunostimulant, NK cell.

Introduction

Immune-mediated diseases are a crucial problem in developing countries. The major causes of human infection are various pathogenic microorganisms in the environment, including viruses, bacteria, fungi, protozoa, and parasites.[1] The immune system is the defence mechanism in the human body, and there are two types: non-specific and specific immune systems.[2] The non-specific immune system is the immune system present from birth, while the specific immune system is an acquired immune system.[3] The non-specific and specific immune systems work together to fight the infection in the body. The immune response consists of various cells and soluble molecules secreted by these cells. The primary cells involved in immune reactions are lymphocytes (B cells, T cells, and NK cells), phagocytes (neutrophils, eosinophils, monocytes, and macrophages), and accessory cells (basophils, mast cells, and platelets).[4] NK cells are lymphocyte cells that are capable of directly killing target cells without pre-sensitization or reliance on the major histocompatibility complex (MHC). These cells are not MHC-dependent and do not interact with target cells via T-receptors (TCR). Certain immunostimulatory compounds enhance the body's defence mechanisms (specifically and non-specifically) and cellular and humoral defence mechanisms.[5]

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Plants can provide certain immunostimulatory chemicals.[6] One herb often used in Indonesia is *Hydrocotyle sibthorpioides* Lam.[6] It is used as an anti-swelling, anti-inflammatory, laxative, antibiotic, fever reducer, detoxifier, and expectorant.[7] Yu and co-workers reported that *H. sibthorpioides* extract produced excellent antitumor effects and demonstrated the ability to influence the immunological function of mice.[8] Moreover, *H. sibthorpioides* has been studied as an immunostimulant, increasing the activity and phagocytosis capacity of macrophage cells, having an anti-inflammatory effect when used topically,[9] reducing TNF-alpha levels, and increasing the activity of NK and CD8 cells from male mice exposed to viral antigen.[10] Also, the ethanol extract of *H. sibthorpioides* has been shown to have a haematopoietic effect on anaemic mice.[11] Furthermore, the safety of *H. sibthorpioides* extract has been investigated in the toxicity tests of LD_{50},[12] SGOT and SGPT,[13] carbon clearance,[14] creatinine clearance,[15] as well as evaluating the histology of mouse liver tissue and kidneys.[16] A novel drug's effectiveness in treating patients is examined in a clinical study after it has first been tested on animals in pre-clinical studies. Clinical trials ensure the effectiveness, safety, and side effects of associated medications that frequently manifest in humans. Medical science justifies using healthy or ill people in trials because it will be beneficial to society to comprehend the effects of the remedy so that it can be utilized in a larger population with greater assurance about its necessary protection.[17] According to previous studies, *H. sibthorpioides* extract, an immunostimulant that enhances NK and CD8 cellular activities, will be effective in destroying different viruses and boosting the body's defense.

Therefore, the aim of the present study was to examine the safety of *Hydrocotyle sibthorpioides* Lam. extract in people during the first phase of a clinical trial.

Materials and Methods

Sources of chemical materials
The chemicals used in the study included 70% ethanol, 96% ethanol, rutin, silica gel 60 F254, Mayer's reagent, Dragendorf's reagent, HCl, metal Mg, $FeCl_3$, $HgCl_2$, $AlCl_3$ (Merck), sodium acetate, an enzyme-linked immunosorbent assay (ELISA) kit, NK, and CD8 cell (BT Lab).

Equipment used
The equipment used included Pyrex glasses, a capsule filler, a blender (Panasonic), a porcelain crucible, a drying cabinet, a mortar and pestle, a rotary evaporator (Ohaus), an analytical balance (Vibra AJ), an ELISA reader, and UV-vis spectrophotometry (Genesys 10 S UV-Vis).

Ethical approval
The ethical approval for this study was obtained from the Ethics Committee, of the Faculty of Medicine, Universitas Andalas, with approval number: 1072/UN.16.2/KEP-FK/2022. The ethical clearance was in accordance with the 64th World Medical Association General Assembly, held in Fortaleza, Brazil, in October 2013.

Collection and identification of plant material
Fresh leaf samples of *Hydrocotyle sibthorpioides* were collected from Jalan Bukit Ngalau, Lubuk Kilangan sub-district, Indarung district, Padang City, West Sumatera, on May 1 and June 30, 2022. Dr. Nurainas from Andalas University Herbarium (ANDA), Department of Biology, Faculty of Mathematics and Natural Science, Universitas Andalas, Padang City, West Sumatera, identified and validated the plant sample. The voucher specimen number is 247/K-ID/ANDA/V/2021.

Preparation of Hydrocotyle sibthorpioides extract
The maceration method was used to prepare the plant extract. One part of powdered *H. sibthorpioides* was combined with ten parts of 70% ethanol solvent in a dark-coloured bottle as a maceration medium. The mixture was stirred occasionally for the first six hours, set aside for 18 hours, and then filtered. The process was repeated twice with the same ratio of powder and solvent. All the filtrates were collected and then evaporated with a rotary evaporator at 40ºC until a thick extract was obtained. The thick extract from the extraction process was dried by freeze-drying.

Formulation of capsule dosage
The dosage to make capsules was 10 mg/kg of human body weight. As a result, the extract in each capsule was 67 mg. All ingredients were weighed according to their concentrations in the formula. *Amylum manihot*, aerosol, and dry extract were ground until homogeneous, and magnesium stearate and lactose were added. The mixture was then put together in capsule shell number two. The composition of each capsule is shown in Table 1.

Standardization of the extract
Hydrocotyle sibthorpioides extract was characterized in both specific and non-specific ways. The non-specific method consists of drying shrinkage with a yield of 5.56%, a total ash content of 2.55 and 0.07% insoluble acid ash. In contrast, the specific way includes organoleptic tests, identity parameters, chemical content tests, thin layer chromatography, and the determination of total flavonoid content.

Table 1: Composition of the capsule

Composition	F1	F2
Hydrocotyle sibthorpioides extract	-	67 mg
Aerosil	2%	2%
Amylum manihot	2%	2%
Magnesium stearate	1%	1%
Lactose	Qs	Qs

Evaluation of Hydrocotyle sibthorpioides powder extract and capsules
The powder was evaluated using flow rate, compressibility, and angle of repose tests prior to being placed within the capsule. The capsules were evaluated using organoleptic tests, weight uniformity tests, and disintegration time tests.

Thin-layer chromatographic separation of Hydrocotyle sibthorpioides extract
In the thin-layer chromatographic test, the eluent or mobile phase used was n-butanol: acetic acid: water (4:1:5). The test solution and sample solution were then spotted on the F254 silica gel plate at the lower boundary, which had been outlined with a pencil. The plate was 10 cm long and 3 cm wide, with top and bottom limits of 1 cm each. After being spotted, the silica plate was placed into the chamber containing the eluent or mobile phase. Earlier, filter paper was used to saturate the chamber. The plate was then taken out and dried before being examined under UV light (366 nm) to form spots using the $AlCl_3$ stain spotter.

Clinical trial procedure
The study used twenty healthy volunteers aged 18 to 35 and divided into two groups. The first group was given preparations without the active substance, while the second group was administered the test preparations. Both groups had blood drawn both before and after the administration of the test preparations. For three days, the tablet was administered once a day.

Collection of serum for NK and CD8 cell tests
After the individuals had fasted for six to twelve hours, blood samples were taken. An aliquot of 5 mL of blood were drawn through the median cubital vein. The puncture site was cleaned with 70% alcohol. The blood was centrifuged for 30 minutes at 3000 rpm, and then the serum was used to test the levels of NK and CD8 cells using the ELISA method. Using the ELISA kit, the protein markers from NK and CD8 cells were determined in plasma cells.

Statistical analysis
The data were analyzed statistically using the simple paired test analytical method. The analysis was conducted with IBM (version SPSS 24).

Results and Discussion

The results of protein concentration in NK and CD8 cells are presented in Figures 1 and 2. The *H. sibthorpioides* collected was cleaned, and washed from impurities with running water, then air-dried until it became dry simplicia. The dried simplicia was crushed using a blender and sifted through sieve no. 48 to extend the surface area of the simplicia to make the process of extraction of the active ingredient more effective and efficient. As a result, the solvent permeates and facilitates easy extraction of the active ingredient. The maceration method was used for the extraction process. This method was chosen since it can employ a lot of samples, is simple, and has no special treatment. In addition, since there is no heating involved, the chemicals in the sample are not broken down. Sample maceration was made using 70% ethanol as the solvent.[18] The use of ethanol as a universal solvent is due to its ability to dissolve polar, semi-polar, or nonpolar compounds quickly.[19] Maceration was conducted for 24 hours with three repetitions using 70% ethanol (1:10). The powder was soaked for the first 6 hours while stirring occasionally and allowed to stand for 18 hours. Maceration was carried out with two repetitions and then filtered using filter paper. The macerated substances were collected and then evaporated using a rotary evaporator to obtain a thick extract.[18]
After obtaining *H. sibthorpioides* extract, the extract was standardized to examine non-specific and specific parameters to obtain a safe and quality-assured extract. The non-specific parameters include drying shrinkage, ash content, and acid-insoluble ash content. Determining the drying shrinkage is to provide a minimum limit or range of the number of compounds lost in the drying process.[18] An examination of the specific parameters of *H. sibthorpioides* was conducted to provide accurate identification and a specific name for the plant. The evaluation results are highlighted in Table 2. The organoleptic test determines the

characteristics of the *H. sibthorpioides* extract, a viscous extract with a distinctive odour, dark brown colour, and bitter taste. The test results are shown in Table 3.

A phytochemical test was conducted on the extract's chemical composition. Hydrocotyle sibthorpioides extract was tested using specified reagents, and the results revealed that the extract included flavonoids, phenolics, terpenoids, and saponins. One of the bioactive compounds in *H. sibthorpioides* extract is a flavonoid that can increase the proliferation of lymphocytes, or T cells. In addition, flavonoids inhibit macrophage cytokine production and reduce T-cell inflammation. Polyphenol molecules also have the potential to increase or decrease innate and adaptive immune responses, depending on their molecular structure. Flavonoids have the potential to prevent and treat inflammatory diseases.[20] Furthermore, the flavonoid group can also act as an immunomodulator, boosting the immune system to ward off attacks by viruses, bacteria, or other microbes.[21]

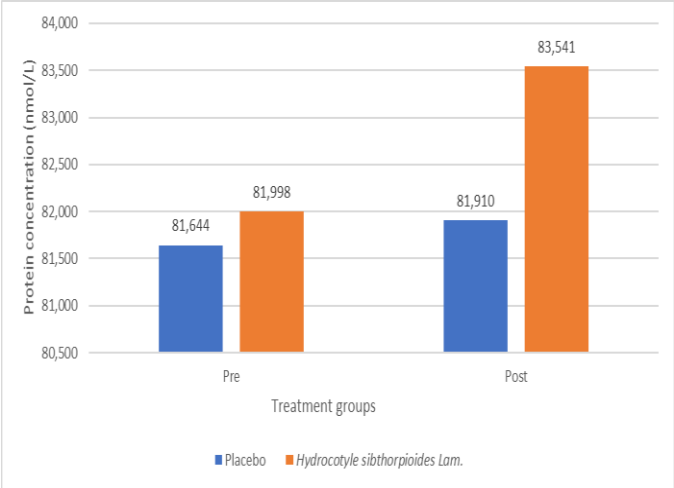

Figure 1: Effect of *Hydrocotyle sibthorpioides* Lam. extract on NK cell protein concentration

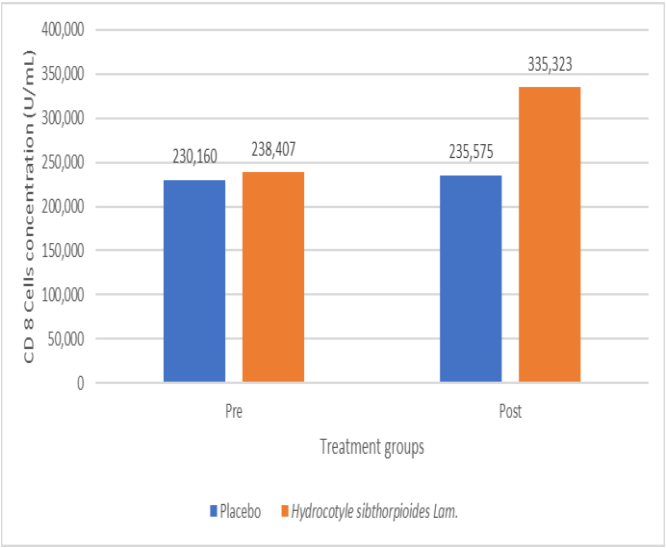

Figure 2: Effect of *Hydrocotyle sibthorpioides* Lam. extract on CD8 cell protein concentration

The sample that was used to examine NK and CD8 cells was serum obtained by centrifuging 5 mL of human blood from the elbow fold. Then, its concentration was measured with the ELISA kit. Infected and undesirable cells in the human body can be effectively eliminated by NK and CD8 cells.[22] Based on the results of the average protein weight of NK cells from several treatments in the placebo group and the test preparations for *H. sibthorpioides* extract capsules, the concentration of NK cells was higher in the group administered with the test preparations compared to the group given the placebo. NK cells play an essential role in non-specific immunity to intracellular pathogens, which recognize and kill abnormal cells and destroy cells containing viruses or neoplastic cells. Interferons, which are often produced and released by cells that are infected with viruses, activate NK cells. NK cells are also fundamental in the natural defence against the growth of cancer cells and various infectious diseases, especially viral infections. The majority of NK cells (95%) function as killer cells, which destroy target cells directly without pre-sensitization or without the assistance of MHC. In addition to being MHC independent, these cells also do not interact with T-cell receptors (TCR).

NK cells play an important role in the natural defence against the growth of cancer cells and various infectious diseases, especially viral infections.[21] They are a component of the innate immune system that acts as a killer (cytotoxic) by secreting lysosomes containing perforin and granzymes that produce the cytokines IFN-γ, TNF-α, IL-5, and IL-13. Along with stimulating macrophages, T cells, and B cells, NK cells also serve as co-stimulators, bridging the gap between innate and adaptive immunity.[21] According to the results, the protein weight of CD8 cells showed that the CD8 cell concentration of the test preparation group was 238,407 U/mL, which was higher than the placebo group's concentration of 335,323 U/mL. Both directly and by the activation of apoptosis, CD8 cells attack target cells.[22] CD8 cells contain abundant azurophilic granules, destroying various tumour, infected, and abnormal cells without prior sensitization.[23] The innate and adaptive immune systems carry out an effective viral response in the host by producing various proinflammatory cytokines and activating T cells, CD4, and CD8 cells. T cells are essential for controlling viral replication, limiting viral spread, and clearing infected cells.

Table 2: Evaluation of *Hydrocotyle sibthorpioides* Lam. powder extract.

Preparatory evaluation test	Result	Condition
Flow rate	8.21 g/s	4-10 g/s
% compressibility	14.3%	11-15%
Angle of repose	32.31º	21-35º

Table 3: Evaluation of *Hydrocotyle sibthorpioides* Lam. capsule.

Preparatory evaluation test	Result	Condition
Organoleptic	According to requirements	Clean, dry, no particles attached to the capsule shell
Weight uniformity	According to requirements	<10%
Disintegration time	2 min 41 second	<15 minutes

Conclusion

The findings of this study reveal that at a dose of 67 mg, *H. sibthorpioides* extract in capsules increase the activities of NK and CD8 cells in humans. As a result, the body's ability to kill invading viruses could be enhanced due to the increased activities of NK and CD8 cells

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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