THE EFFECT OF *HOMALANTHUS POPULNEUS* (GIESEL.) PAX. EXTRACT IN EXPRESSION OF T-CELL RECEPTOR: INHIBITION STUDY OF HIV INFECTION

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**ABSTRACT**

**Objective:** The aim of this study is to analyze the effect of *Homalanthus populneus'*s extract toward the expression of CD4 and CD8 which both are important in body's defense mechanism against HIV.

**Methods:** Leaves and barks of *H. populneus* were extracted with 70% ethanol. Freeze dry method had been used in order to get the final extract. PBMCs were extracted and it has been used for CD4 and CD8 expression test using flow cytometry. In order to analyze the effect of the extract to the HIV type 1, gp41 and gp120 expression was tested using taliycytometry and using enzyme-linked immunosorbent assay, respectively.

**Results:** This study reported that *H. populneus'*s extract reduced the expression of CD4 receptor in both peripheral blood mononuclear cell (PBMC) and T-lymphoblast cell line (CEM). In contrast, this extract increased CD8 expression in PBMC. It was also able to reduce the percentage of protein gp41 and gp120 in GEM cultures.

**Conclusion:** Those results show that *H. populneus'*s extract is potentially developed as an HIV drug from Indonesia. However, further study needs to be done including analyzing the effect of variety of concentrations and also exposure periods.

**Keywords:** *Homalanthus populneus*, CD4, CD8, HIV.

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**INTRODUCTION**

Prostratin is a secondary metabolite of plants which is endemic in the Samoa Islands (South Pacific Ocean Islands) named *Homalanthus nutans* [1,2]. Prostratin can activate the expression of HIV-1 in cells infected with HIV by increasing viral replication but not followed by an increase in cell division; thus, these cells produce new viruses that can be recognized by immunocompetent cells [3]. Prostratin also inhibits the expression of chemokine receptors in helper T cells, which are the entry points for viruses into lymphocytes [4,5]. In Indonesia, there are plants which have same genus category as *Homalanthus nutans*, called *Homalanthus populneus*. This plant is spread abundantly in Indonesia. In the previous studies, it was known that this plant contained prostratin [6] and was able to reduce the expression of cluster of differentiation (CD4) receptors up to 82.84% in normal cells [6]. Prostratin from *H. populneus* is potential to be developed as an anti-HIV drug from Indonesia. Therefore, it is necessary to test to determine the effectiveness of this local prostratin in inhibiting HIV infection by inhibiting the expression of T helper. This study also wants to analyze the effect of ethanol extract of *H. populneus* to the expression of HIV by calculating its capsid proteins which are gp120 and gp41.

**MATERIALS AND METHODS**

**Reagents**

Ethanol extracts of leaves and bark of *H. populneus* (Giesel.) Pax. from the previous study had been used in this research. The concentration of prostratin had been measured with high-performance liquid chromatography method. Monoclonal antibody to CD4 and CD8 was purchased from Thermo Fisher Scientific (Waltham, USA). HIV-1 gp41 and gp120 antibodies were purchased from LSBio (LifeSpan BioScience, Inc., North America).

Peripheral blood mononuclear cell (PBMC) preparation

PBMCs were isolated from 15 ml blood of healthy donors using centrifugations. Roswell Park Memorial Institute (RPMI) was added to the blood sample with ratio 1:1. Ficoll-Paque Plus (Sigma-Aldrich, Darmstadt, Germany) was utilized to collect PBMC from blood. Before blood mixed with Ficoll, RPMI was added until 30 ml (Sigma-Aldrich, Darmstadt, Germany). Comparison of Ficoll Plus and blood sample was 3 ml:8 ml. After that, it was centrifuged for 20 min, 400 D, at 18°C. The formed buffici coat was separated and then mixed with RPMI up to 13 ml. It was then centrifuged for 10 min at 18°C and at 400 D. The deposited pellets were mixed with 4 ml complete medium. Finally, lymphocyte cells were counted using Neubauer hemocytometer method.

PBMCs proliferation test

PBMC proliferation test was carried out by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PBMC cells that were mixed with a complete medium (2 ml of penicillin-streptomycin, 0.5 ml of fungison, and 80 ml of RPMI, all were purchased from Sigma-Aldrich, Darmstadt, Germany) were incubated for 24 h at 37°C and 5% CO2 in a well microplate 96. Cell density in each well is 1×104 cells/100 ml/well. Six different concentrations of the ethanol extracts of *H. populneus*’s barks (B) and leaves (D) were used in this study (0.125, 0.5, 1, 62.5, 125, and 250 µg/ml). At the end of the incubation period, 10 ml of 5 mg/ml MTT (Invitrogen, Thermo Fisher Scientific, Waltham, USA) was added to each well. Cell suspension was then incubated at 37°C 5% CO2 for 4 h. The reaction was stopped by adding 1% Safety Data Sheet (Invitrogen, Thermo Fisher Scientific, Waltham, USA) in 0.1 N HCl (Sigma-Aldrich, Darmstadt, Germany) as much as 10 ml/well. The absorbance value was read using enzyme-linked immunosorbent assay (ELISA) reader (LABOMED, Los Angeles, USA) with a wavelength of 550 nm.
Transfection PNL43 plasmid
The first solution was lipofectamine, 7.5 µl of lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, USA) was dissolved in 250 µl medium Opti-MEM (Invitrogen, Thermo Fisher Scientific, Waltham, USA). The second solution, the master mix DNA, 5 µg of DNA was dissolved with 250 µl of Opti-MEM medium. The first and second solutions were mixed and incubated for 5 min. This cDNA-lipid complex was added to cell culture with green fluorescent protein (Invitrogen, Thermo Fisher Scientific, Waltham, USA) on flask 25, incubate 3 days; then, it was observed under a fluorescence microscope, whether the cell is fluorescent (transfection successful) or not (transfection fails).

ELISA
105/100 ul cells with PNL43 were planted in in plate 96-well with six replications and incubated for 1 day. Three different concentrations of the ethanol extracts of H. populneus's leaves were used in this study (7, 11, and 15 µg/ml). Bark extract had been chosen rather than leave extract since bark extract contains higher concentration of prostratin. After adding the extract, the cells were incubated at 37°C with 5% CO₂ for 3 days. The ELISA test was then carried out using gp 41 antibodies.

Flow cytometry and Tali Image-based Cytometer
The PBMC cells were incubated on the well microplate 24 with a density of 45×10⁶ cells/100 ml/well. Three different concentrations of the ethanol extracts of H. populneus's leaves were used in this study (7.8, 15.6, and 31.2 µg/ml). Cells that have been given treatment are then incubated again for 24 h. After that, it was harvested using phosphate-buffered saline (PBS) and centrifuged gradually with a speed of 2000 rpm for 25 min at 4°C. Harvested cells were suspended into PBS to 0.5 ml in a 1.5 ml microtube. After that, it was added with 10 µl antibodies (gp120, mixture of CD4 and CD8), then incubated for 15 min in the darkroom. Then, PBS (Thermo Fisher Scientific, Waltham, USA) was added and it was centrifuged again at 1500 rpm for 5 min. The pellets were added with 1 ml of fluorescence-activated cell sorting flow. Then, one by one measured the percentage of CD4 and CD8 counts using flow cytometry [7] (Becton Dickinson, Calif., USA). To detect the concentration of gp120, Tali Image-based Cytometer was utilized.

RESULTS
Ethanolic extract of H. populneus induces PBMCs replication
PBMCs were obtained from women aged 21/22 years who had no previous history of severe illness. In this study, from 12 ml blood, 6.5×10⁶ cells/ml PBMC were obtained. PBMCs from collected blood must be isolated immediately to prevent blood clots. Proliferation test was carried out using PBMC as much as 1×10⁶ cells/ml PBMC. Ethanolic extract of H. populneus was added to cell culture with green fluorescent protein (Invitrogen, Thermo Fisher Scientific, Waltham, USA) on flask 25, incubate 3 days; then, it was observed under a fluorescence microscope, whether the cell is fluorescent (transfection successful) or not (transfection fails).

Table 1: Prostratin concentration in ethanolic extracts of leaves and bark of H. populneus and percentage of PBMC proliferation effects caused by it

| Test and extract type       | Concentration (µg/ml) | Level of prostratin (µg/ml) | Average proliferation (%) |
|-----------------------------|-----------------------|-----------------------------|---------------------------|
| PBMCs proliferation test by adding leaves extract | 250 | 4.9 | 125.9 |
|                             | 125                   | 2.5                         | 81.6                      |
|                             | 62.5                  | 1.2                         | 53.6                      |
|                             | 1                     | 0.3                         | 17.6                      |
|                             | 0.5                   | 0.2                         | 7.4                       |
|                             | 0.125                 | 0.02                        | 0.8                       |
|                             | 250                   | 19                          | 143                       |
|                             | 125                   | 9.5                         | 95.3                      |
|                             | 62.5                  | 4.7                         | 70.7                      |
|                             | 1                     | 1.2                         | 27.6                      |
|                             | 0.5                   | 0.6                         | 13.5                      |
|                             | 0.125                 | 0.1                         | 3.7                       |

H. populneus: Homalanthus populneus; PBMC: Peripheral blood mononuclear cell

The stopper was added to dissolve the formazan crystals that were formed so the cultures that contain many living cells will turn to be purple. The colorimetric results of this optical density correspond to the number of living cells. This method is very sensitive. The proliferation test results in this study are shown in Fig. 1.

Table 1 shows the specific level of prostratin which causes the proliferation happened. It can be seen that the bark extract contains higher concentration of prostratin compared to leaves extract; therefore, only bark extract that was used for the next tests.

Ethanolic extract of H. populneus downregulated CD4 and upregulated CD8
Cytometric flow readings are scattergrams that are the result of forward scatter and side scatter. Gating was done to select more specific cell groups based on the expression of CD4 and CD8. Before test, 5 µl/ml anti-CD3 was added. The presence of this CD3 receptor will interfere the bonding between T-cell receptor (TCR) and antigen-presenting cell (APC), so anti-CD3 is needed. No disruption of TCR-APC bonding will accelerate the process of interleukin-2 (IL2) formation in PBMC cultures. IL2 itself plays a major role in regulating the process of proliferation and differentiation of T cells.

Fig.2 shows that there was a possibility that the PBMC culture contained not only lymphocytes but also monocytes. Analysis using CD4 and CD8 monoclonal antibodies showed that the number of lymphocyte cells from the total cells obtained was 71.3% in cell control and 70.4% in solvent control. The number of lymphocyte cells in cell control and solvent control was not much different; this showed that DMSO solvents can not only work on to PBMC cells. Hence, it can be estimated that the decrease in the percentage of CD4 or CD8 receptors not caused by solvents which in this study was DMSO.

Ethanolic extract of H. populneus downregulated protein gp41 and gp120
In this test, previously labeled antibodies, gp41 (for HIV-1 capsid protein) was used. Test was carried out on cell cultures that were positively transfected by PNL4-3 plasmids. The concentrations of bark extract that was used in this test were 7, 11, and 15 µg/ml. The results of the Tali Image-based Cytometer test are shown in Fig. 3.

Furthermore, to quantify the gp120 expression, ELISA method was used. Before coating to the well plate 96, 1% formaldehyde was added to the culture cell, the purpose of which was to lyse the cell wall so that the gp120 antibodies could bind to the gp 120 antigen within the cell. After that, cells in well plate 96 were incubated overnight at 40°C to give sufficient time so that all the gp120 antigens that have been coated can become available for binding to the antibodies. After washing had been done, the anti-gp120 monoclonal antibodies were added. After 2 hours, the plate was again washed and a colorimetric solution was added to the wells. The absorbance at 490 nm was measured using a plate reader. The results of this ELISA test are shown in Fig. 4.
Fig. 1: The average percentage of peripheral blood mononuclear cells proliferation by adding ethanolic extract of leaves (a) and bark (b) of Homalanthus populneus with concentrations of 0.125, 0.5, 1, 62.5, 125, and 250 µg/ml and controls

Fig. 2: The results of flow cytometry using cluster of differentiation (CD8) and CD4 antibodies on peripheral blood mononuclear cell without extract, (a) control cell, (b) solvent only, and with ethanol extract of Homalanthus populneus’s bark, (c) 7.8 µg/ml, (d) 15.6 µg/ml, and (e) 31.2 µg/ml

Fig. 3: Percentage of gp41 using Tali image-based cytometer test with two replications

DISCUSSION

MTT is used to qualitatively viability cell based on mitochondria metabolic activity used by the cells. The process of MTT assay is based on transformation of tetrazolium salt (MTT) into formazan crystals. MTT, dissolved in PBS, will be absorbed to the viable cell by the existing gradient concentration of solution inside and outside the cell (solution in the cell is more thick). Next, yellow-colored tetrazolium salt is reduced by complex enzyme of succinate dehydrogenase, which is active when metabolic process occurred in mitochondria [3]. The aim of the stopper is to dissolve the formed formazan crystals so that the culture contains abundant viable cell with purple color [3]. Increasing percentage of proliferation (number) of PBMCs is comparable directly with increasing concentration of tested compound. The results showed that tested compound did not cause death against PBMC, but instead, it induced PBMC proliferation.

Table 2: The ELISA test results using gp 120 antibodies with three different concentrations of bark extract (7, 11, and 15 µg/ml)

| Concentration of the extract (µg/ml) | Presentation of gp 120 protein |
|-------------------------------------|-------------------------------|
| 0                                   | 92                            |
| 7                                   | 81                            |
| 11                                  | 77                            |
| 15                                  | 72                            |

ELISA: Enzyme-linked immunosorbent assay

Table 3: Prostratin levels at the concentrations of the ethanol extracts of leaves and bark of H. populneus based on previous HPLC data

| Type of extract | Konsentration (µg/ml) | Level of prostratin (µg/ml) |
|-----------------|-----------------------|----------------------------|
| Leaves extract  | 7.8                   | 0.1                        |
|                 | 15.6                  | 0.3                        |
|                 | 31.2                  | 0.6                        |
| Bark extract    | 7.8                   | 0.6                        |
|                 | 15.6                  | 1.2                        |
|                 | 31.2                  | 2.4                        |

H. populneus: Homalanthus populneus, HPLC: High-performance liquid chromatography
HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CCR5. This receptor is attached to each other but has different functions in the mechanism of HIV infection. Gp120 and gp41 are both capsid proteins of the HIV whose position is very important and related to one another. When this bond is formed, the gp120 stretches and provides room for the HIV genetic material into the host cell. Hence, the performances of gp120 and gp41 are very important and related to one another. Therefore, these two types of proteins can be linked as markers of the presence of the HIV in the host cell.

CONCLUSION

Plant extract of *H. populneus* (Giesel) Pax. can reduce the percentage of helper T cells and increase the percentage of cytotoxic T cells in human PBMC. The results of testing using cell cultures showed that administration of ethanol stem extract of the bark of *H. populneus* was able to reduce the percentage of CD4 expression and reduce the amount of HIV formed in positive cell cultures infected with HIV. Therefore, it can be concluded that this plant is potential to be an effective source of prostratin. Further research needs to be done to obtain optimal concentrations of this extract as a potential anti-HIV drug from *H. populneus*.

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