Immunomodulatory Effect of Ethyl Acetate Extract of Permot (*Passiflora Foetida* L.) Leaf against the Secretion of Antibody and Delayed Type Hypersensitivity *in Vivo*

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Abstract

The aim of this study was to investigate the immunomodulatory effect of ethyl acetate of extract of *Passiflora foetida* L leaves (EAEPL) on hypersensitivity response and antibody titer of immune cells. Hypersensitivity response test evaluated the swelling of the feet of mice, while antibody titer test evaluated the hemagglutination. Examination of antibody titer was performed in 5 groups of 25 wistar rats. All the test animals, except control group, induced by SRBC on the 3rd day intraperitoneally. The extract was given for 7 days. The hypersensitivity response test was performed in 5 groups of 25 mice (Balb/c). The groups, except control group, were given antigen (SRBC) on the 3rd day intraperitoneally and on the 7th day were administered intraplantarly. The thickness of the soles of the feet was measured using a plethysmometer before and after injecting the antigen at T4, T24 and T48. The data were processed statistically by Kruskal-Wallis then advanced test by Mann-Whitney. The group of negative control against the groups of EAEPL was significantly different ($p < 0.05$). The antibody titers of the groups given the extract were higher than the antigen-induced group without the administration of the extract. The results show that the groups of EAEPL can increase antibody secretion. The test of hypersensitivity response showed that the group of negative control to EAEPL groups showed significantly different ($p < 0.05$). The results showed that the swelling of the feet of mice decrease at T48. This research concludes that ethyl acetate extract of EAEPL has the immunostimulatory effect.

Keywords: Immunomodulatory, *Passiflora Foetida* L, Delayed Type Hypersensitivity

Introduction

Immune system is a body mechanism which is used for protection against the risk from various substances in the environment. To maintain our immune system, immunomodulator is needed, in which it is a compound or medicine that triggers immune system by inducing or increasing the activity of immune system components [1, 2].

Immunomodulator functions to improve immune system by stimulating (immuno stimulant) or suppressing/normalizing abnormal immune reaction (immunosuppressant) [1, 2]. Hypersensitivity reaction is the manifestation from excessive immune response towards an excessive

antigen and trigger tissue damage. Hypersensitivity type IV, which is known as a delayed hypersensitivity, is a type of hypersensitivity which stimulates interleukin (cytokine) which is secreted by T cell and macrophages in killing the antigen. Macrophages which are activated can be differentiated into giant cell and developed into granuloma hypersensitivity, DTH creation which is marked by the formulation of granuloma [3].

Therefore, immunomodulator agent has been widely developed as a new alternative to cure the disease. One of the plants which contains medical compound is *Passiflora*. *Passiflora*

genus consists of more than 400 species; one of them is *Passiflora foetida* L. or known as Permot. The main components of Permot are alkaloid, phenol, flavonoid glycoside also components of *cyanogenic* and *passiflorine*, and *polyketide* and *alfa-pyrones* [4, 5].

It has been known that Permot has the ability as antioxidant with IC₅₀ value is 1, 0004 mg/ml, which has been tested by hypoglycemic activity [6]. The immunomodulator effect test from Permot's ethanol extract showed the immunomodulator activity for the dose of 200, 400, and 600 mg/BW [7]. According to the explanation above, there has been no scientific data about the immunomodulator activity test of ethyl acetate extract in Permot by using parameter for antibody titer secretion and delayed type hypersensitivity (DTH).

For that reason, this study carried out a research about immunomodulator effect of Permot leaves' ethyl acetate extract with parameter for antibody titer secretion and DTH. The results of the research can add more reference of the immunomodulator use from plants to prevent various diseases and add inventory of medical plants which function as immunomodulator.

Material and Method

The material used was Permot leaves from Makassar Indonesia, antigen of sheep red blood cell (SRBC), Phospat Buffered Saline (PBS), rat, mouse, wheel plate, and plethysmometer.

Processing the Sample

The sample used from Makassar, Indonesia. The sample was cleaned from any dirt by water flow and dried by indirect sunlight.

Sample Extraction

The 500 gr of sample was stored in maceration container. Then, the ethyl acetate solvent was poured until the sample was submerged, and it was left for 1 x 24 hours. After that, the macerate was filtered and the waste was macerated again. The maceration product was collected and evaporated by rotavapor to get thick extract [8]. Then, it was dried freeze to make it into a dry extract.

Suspension of Sheep Red Blood Cells (SRBC)

The fresh SRBC which had been given anticoagulant was centrifugated by the speed of 1500 rpm, to separate the plasma from the red blood cells. The upper layer containing plasma was taken away by Pasteur pipet, whereas, the bottom layer consisting of red blood cells deposit was added by PBS solution pH 7,2 three times of remaining SRBC volume.

The tube was flipped back and forth slowly until the SRBC was homogeneously suspended, to be then re-centrifugated. This procedure was repeated until the upper part was totally colorless and clear. The clear upper layer was thrown away and the bottom layer was the result of SRBC 100% suspension. The SRBC 100% suspension was taken by 1 mL and added into PBS with the same volume so it was resulted in suspension of SRBC 50%. To get suspension of SRBC 5%, 1 mL of SRBC 50% suspension was pipetted and added by PBS ad 10 mL. For suspension of SRBC 10%, 2 mL SRBC 50% suspension was pipetted and added by PBS ad 10 mL [9].

Antibody Titer Test

This research used 25 male rats (150-200gr) which were divided into five groups. On the first day, every rat was given treatment of test preparation except group I and II for seven days orally. Group I (control group) was left with no treatment, while group II was given SRBC 5% without given the extract. On the third day, group II, III, IV, and V were induced by SRBC 5% v/v for 1 mL intraperitonially. The blood was taken on the day 8th to test the antibody [10].

The blood sample taken after the therapy was let to freeze or clot in the room temperature for 1-2 hours, to be then centrifuged by the speed of 1500 rpm for 10 minutes and the serum (supernatant) was taken. For the dilution, 1/1 was pipetted for 50 µl animal test serum and added by 50 µl PBS in wheel plate.

For ½ dilutions, 50 µl dilution serums 1/1 was pipetted and added by 50 µl PBS in wheel plate. While for ¼ dilution, 50 µl dilution serum ½ was pipetted and added by 50 µl PBS in wheel plate. Then, the serum was diluted orderly became 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 with 50 µl PBS, to be then in each wheel plate was added by 50 µl suspension of SRBC 5% v/v

and stirred evenly for 5 minutes, then incubated in 37° C for 60 minutes and left overnight in room temperature. The observation was to see the highest dilution from the blood serum of rat which still could agglutinate the SRBC [11].

Delayed Type Hipersensitivity Test

The research used 25 male mice (20-30 gr) which were divided into five groups. Group I (control group) was left with no treatment, group II was given SRBC 5% without given the extract, while group II, IV, and V were given the extract by the dose of 0.4 g/kg BW, 0.8 g/kg BW, and 1.2 g/kg BW consecutively. The extract was given for 7 days. On the third and seventh day, the antigen was given. The foot thickness was measured by plethysmometer before and after the antigen injection in T4, T24, and T48 hours.

Results and Discussion

Immune system is a body mechanism used for body protection against the risk from various substances in the living environment. To protect the immune system, immunomodulator is needed. Immunomodulator is a substance or medicine that can modulate the function and activity of immune system.

Antibody Titer

The research used hemagglutination test method which was done by sensitizing SRBC

as antigen to the serum that was diluted gradually. Hemagglutination was considered positive if the whole or most part of the concave surface of the bottom plate was covered by SRBC evenly. The antigen used was SRBC which had high antigenic so the formed antibody was easily detected by using agglutination reaction [9].

The measurement of antibody by using hemagglutination method can be seen in Table 1. The measurement result after being transformed is shown in Table 2. The observation result of antibody agglutination titer showed the highest titer in the extract group (EAEPL) in the dose of 400 mg/kgBW and 600 mg/kgBW which were 1/512 (3,816). The data of antibody titer test which had been transformed by the formula of $\{2^{\log(\text{Titer}) + 1}\}$ was then analyzed by using Kruskal-Wallis analysis test.

The analysis result showed the significantly different result which meant that there was a different antibody titer between treatment group and group which was only induced by antigen ($p < 0.05$). It showed that Permot extract had the ability to increase the immune system. The group which was only induced by antigen and the group control showed the difference ($p > 0.05$), which showed that SRBC was successfully triggered immune system.

Table 1: Antibody Agglutination Titer by using Hemagglutination Method

Replication	Groups of Rat				
	Control	SRBC 5% v/v	EAEPL 200 mg/kgBW	EAEPL 400 mg/kgBW	EAEPL 600 mg/kgBW
1	1/32	1/64	1/128	1/512	1/512
2	1/32	1/64	1/128	1/512	1/512
3	1/64	1/64	1/128	1/128	1/512

Table 2: Antibody Agglutination Titer after Transforming by $\{2^{\log(\text{Titer}) + 1}\}$ Formula

Replication	Groups of Rat				
	Control	SRBC 5% v/v	EAEPL 200 mg/kgBW	EAEPL 400 mg/kgBW	EAEPL 600 mg/kgBW
1	2.010	2.612	3.214	3.816	3.816
2	2.010	2.612	3.214	3.816	3.816
3	2.612	2.612	3.214	3.214	3.816
Average	2.210	2.612	3.214	3.615	3.816
DS	0.347	0	0	0.347	0

Slow Type Hypersensitivity Test

This research used slow type hypersensitivity test that appeared after the body was exposed with antigen in more than 24 hours.

This reaction was chained by contact of T cells which were sensitization with appropriate antigen, so T cell released cytokine which attracted and stimulate

macrophages to release inflammation mediators to the injection site and triggered an inflammatory reaction. In the next exposure around 8-12 hours after being activated, the antigen induced inflammation

and effector response where Th cell which secreted IFN- γ that activated macrophage [1, 2]. The measurement result from treatment group can be seen in Table 3.

Table 3: The Average Change in the Volume of Feet of Mice

Groups of Mice	Time observing the volume of the feet of mice			
	Initial volume + DS	T4 + DS	T24 + DS	T48 + DS
I (Na CMC 1% b/v)	0.15 $\pm$ 0.05	0.15 $\pm$ 0.05	0.15 $\pm$ 0.05	0.15 $\pm$ 0.05
II (SRBC 10% v/v)	0.14 $\pm$ 0.02	0.25 $\pm$ 0.01	0.25 $\pm$ 0.01	0.19 $\pm$ 0.005
III (EAEPL 0.4 g/KgBW)	0.11 $\pm$ 0.001	0.38 $\pm$ 0.08	0.14 $\pm$ 0.04	0.11 $\pm$ 0.005
IV (EAEPL 0.8 g/KgBW)	0.14 $\pm$ 0.01	0.24 $\pm$ 0.11	0.18 $\pm$ 0.01	0.14 $\pm$ 0.02
V (EAEPL 1.2 g/KgBW)	0.14 $\pm$ 0.02	0.25 $\pm$ 0.02	0.20 $\pm$ 0.03	0.14 $\pm$ 0.02

The Table 3 describes the average measurement result from foot volume change at T0, T4, T24, and T48 for each treatment group after given the antigen. The table shows that after the antigen induction, group II, III, IV, and V had increased foot volume. It indicated that the antigen was successful in stimulating the immune response.

The decreased foot volume at T-24 occurred in group II and IV, while it was not shown in group II. It means that the extract given in various doses could decrease the edema volume in rat's foot compared to other groups which was only induced by antigen without given any extract. During the T-48 observation, group II showed a decrease of edema volume although it was still relatively smaller than the decrease in group III, IV, and V. Group III, IV, and V during T-48 observation had shown insignificant edema volume by T0 ($p>0.05$). On observations of T-48, edema volume of group II had not shown the initial condition (T0).

Based on slow type hypersensitivity reaction theory (type IV), the increased foot volume did not occur for 6-12 hours and reached maximum intensity after 24-72 hours [3]. Thus, in this study the groups which were given the extract showed higher ability to stimulate the immune system than the group without extract (group II). The measurement result of foot volume change at T48 was analyzed using Kruskal-Wallis analysis test

which showed the significantly different result ($p<0.05$). It meant that there was a difference among each treatment group. The difference of titer among treatment groups was shown by the following Mann-Whitney analysis test. Based on the result of Mann-Whitney analysis test, for each extract group, group with EAEPL dose of 0.4 g/kgBW and 1.2 g/kgBW that indicated that the difference was not significant ($p<0.05$).

However, group with EAEPL dose of 0.8 g/kgBW showed a significant difference ($p>0.05$). It represented that statistically there was a difference among each extract dose. Immunomodulator extract was indicated coming from phenolic compound content which had antioxidant activity [12, 13].

Conclusion

In this study, ethyl acetate extract of *Passiflora foetida* L leaves showed the ability to secrete antibodies and provide a better hypersensitivity response ($p<0.05$) compared to the group which was only induced by antigen (SRBC). The ethyl acetate extract of *Passiflora foetida* L leaves had an effect as an immunostimulator.

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