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Immunomodulatory Activity of Pacar Air (*Impatiens balsamina* Linn.) Herb Ethyl Acetate Fraction in Mice

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Abstract : The aim of this studyis to evaluate immunomodulatory effect of ethyl acetate fractionof pacar air (*Impatiens balsamina* Linn.) in Balb/C mice. The assessment of immunomodulatory activity on specific and nonspecific immunity were studied by titerantibody, Delayed Type Hypersensitivity (DTH), carbon clearance test and organ index. Ethyl AcetateFraction of Pacar Air (EAFPA)was administered orally at the dosage levels of 125 mg/kgbw and 250 mg/kgbw in Balb/C mice. In order to induce immunosuppresion in mice, methylprednisolone is used (15 mg/kgbw, i.p), and levamisole (50 mg/kgbw, p.o) as immunostimulating agents, and carboxymethylcellulose (1%) as normal control.Results of present study clearly indicate that EAFPA 125 mg/kg bw and 250 mg/kg bw shows potentiation of immunosuppressant effecton specific and nonspecific immunity.On specific immunity, highest decrease of footpad thickness was due to 125 mg/kg bw of EAFPA(7.0026 ± 2.3496) in DTH response ($p<0.05$) after 24 hrs challenge. Primary and secondary titer antibody value were (5.0939 ± 0.5037) and (5.9368 ± 0.5037) respectively.Nonspesific immunity had its carbon elimination rate lower than the normal control group with phagocytic index values ($k<1$), were 0.86 and 0.74 respectively and organ index, shows a decrease in spleen index compared to normal control group which is significant($p<0.05$).EAFPA at doses of 125 and 250 mg/kg bw hasimmunosuppressant effectwith an effective dose of 125 mg/kg bw.

Keywords :*Impatiens balsamina* Linn., immunomodulator,Delayed Type Hypersensitivity, titer antibody, carbon clearance test, organ index.

Introduction

Immunomodulator is a compound that can restore the imbalance of the immune system by stimulating and improving immune system function.The balance of the immune system needs to be maintained to keep the body healthy. The immune system is closely related to the presence of antibodies. Antibodies are immune system proteinswhich are humoral form of immunoglobulin produced by B cells were fixed to the antigen[1]. The immune system is a system that is very important for the body to prevent and fight various diseases[2].

The active compounds contained in the plant which has the effect of influencing the immune system in the last decade ranging widely applied as a "immunotherapy", which is a method of treatment that combines conventional treatment with immune therapy to gain maximum treatment against various diseases[3]. According to the research that has been done, one of the plants useful as immunomodulator is pacar air (*Impatiens balsamina* Linn.)[4]. *Impatiens balsamina* Linn. has been widely used to treat rheumatism, isthmus, broken

bones, superficial infection, inflammation of the nail, antifungal, antibacterial, antipruritic, antianaphylaxis and antitumor activity[5].The aim of this study is to evaluate immunomodulatory effect of ethyl acetate fraction of pacar air (*Impatiens balsamina* Linn.) inBalb/C mice.

Experimental

Plant Material Collection and Extract Preparation

*Impatiens balsamina*plantswere collected from PutriDaraNante Street, Pontianak, West Borneo.Plant determination were performed at Biology Laboratory of Mathematics and Natural Sciences, University of Tanjungpura, Pontianak.Shade dried leaves and stems were crushed and extracted with96% ethanol and fractionated with ethyl acetate. Extracts and fractions were concentrated by vacuum distillation. Fractions were dissolved in 1% CMC-Na and used for further study.

Animals Used

Balb/C albino mice (Approx 20 to 25 gm) were procured from GadjahMada University, Yogyakarta.

Experimental Design

Animals were divided into different groups each containing 7 animals.

Group I –Negative Control, 1% CMC-Na suspension
Group II - Standard, Levamisole, 50 mg/kg b.w, p.o
Group III - Standard, Methylprednisolone, 15 mg/kg bw, i.p
Group IV, V- EAF 125, and 250 mg/kg b.w, p.o

Specific Immunity Assay

Antigens

Sheep Red Blood Cells (SRBC) was used as antigens for specific immunity assaywhich collected from Biofarma, Bandung, Indonesia. SRBC were washed 3-4 times with large quantity of sterile and pyrogen free saline[6].

Delay Type Hipersensitivity

Antigen Challenge

On 0^{th} day, all groups were sensitized with 0.1 ml/10 g bw of SRBC, i.p. On 6^{th} day prior to injection, right hind footpad thickness was measured with digital calipers. Then animals were challenged by injecting 1% SRBC (0,05 ml) into the right hind footpad. On 7^{th} and 8^{th} day footpad thickness was again measured. Difference between prior and post challenge footpad thickness was reported as DTH response.

Titer Antibody Response to SRBC

Experimental design was done same as mentioned in Delayed typehypersensitivity model. On 5^{th} day before challenge, blood was withdrawn from tail vein of each animal. Blood was centrifuged, and serum was separated. Serial two fold dilutions were made i.e. 50 µl of serum was added to 1^{st} well of 96-well micro titre plate containing 50 µl phosphate buffer saline. To this 1% SRBC (50 µl) dissolved in phosphate buffer saline was mixed. From 1^{st} well 50 µl of dilutedserum was added to 2^{nd} well containing 50 µl phosphate buffer saline and 50µl 1% SRBC. Suchdilutions were done till 12^{th} well. Plates were incubated at 37ºC for 1 hr. Highest dilution that has shown visible agglutination was considered as haemagglutination antibody titre[7,8]**.**

Nonspecific Immunity Assay

Antigens

Pelican carbon ink B17 was used as antigens for nonspecific immunity assay

Carbon Clearence Assay

All the animals were treated are above from day 0 to day 7. On 8th day of treatment animals of the entire group received an intravenous injection (0,1 ml/10 g bw) of pelican carbon ink B17 suspension (inkubation at 37°C for 24 hours before used). Blood samples were collected from tail vein to bloodtube at an interval of 4;8;12;16 and 20 min after the injection of ink suspension. Amount of 20 µL of blood sample was dissolved in 2 ml of 1% acetat acid solution to lyses the erythrocytes. %transmittance of these samples was measured at 675 nm using spectrophotometer. Rate of carbon clearence and phagocytic index of treated group animals were compared with the control group animals. The graph for absorbance versus time was plotted for each animal in respective test groups and phagocytic index (PI) was calculated using formula:

$$Phagocytic\ index\ (PI) = \frac{Ksample}{Kcontrol}$$

Where KSample represent the slope of absorbance versus time curve for extract-treated sample and KControl represent the slope of absorbance versus time curve for control.

Organ Index

On the 8th day after administration of the test substance, mice were sacrificed. Liver and spleen were isolated and weighed. Organ index expressed per body weight of each mice and found the significance of changes to the organ index control group.

Statistical Analysis

Values are expressed as mean ± standard deviation (SD), n=5mice in each group.Statistical analysis was performed with one-way analysis of variance (ANOVA). All analysis and comparison were evaluated at 5 % ($P< 0.05$) level was considered statistically significant.

Results and Discussion

Phytochemical Assay

Phytochemical screening showed that ethyl acetate fraction of *Impatiens balsamina*extraction had more secondary metabolite compounds. EAFPA revealed the presence of the following classes of chemical compounds: polyphenols, flavonoid, steroid/triterpenoidandquinone.

Delayed Type Hypersensitivity

In the present investigation, SRBC induced DTH reaction was used tostudy the effect of EAFPA on cell mediated immunity. DTH is an antigen specific and mediated by T cells rather than antibody. T cells are required to initiate the reaction. Activation of T cells releases lymphokines, which lead to activation and accumulation of macrophages, increases vascular permeability, induces vasodilatation, and produces inflammation. It also boosts phagocytic activity and increases concentration of lytic enzymes for more effective killing, ultimately results in increased footpad thickness in immunized animals. General characteristics of DTH are an invasion of immune cells at site of injection and induction became apparent within 24 to 72 hrs [9,10-12].DTH response decreases after 48 hrs in all EAFPA groups and significant as compared to control groups.Potentiation of DTH response was observed in antibodies are immune system proteins which are humoral form of immunoglobulin produced by B cells were fixed by the antigenmethylprednisolone treated animals ($p<0.05$) because it has inhibit T cells in proliferation in immune system. Levamisole, a standard immunomodulatory drug, has shown maximum potentiation of DTH response ($p<0.05$). Decrease in paw edema after 24 hrs of challenge was observed in all EAFPA treated groups when compared to control. It may be

concluded that EAFPAare unable to stimulate the macrophages function to stimulate T cell for thehypersensitivity reaction in the immunized animals. The results are showed in**Table 1**.

Table 1.EAFPA effect on DTH response using SRBC as an antigen in mice

Treatment	Dose (mg/kg bw)	Δ footpad thickness (%)	
		24hrs	48hrs
Control	-	21.2223 ± 2.6368	22.7875 ± 2.1223
Levamisole	50	33.5885 ± 4.5715^{x}	27.6116 ± 3.7470^{x}
Methylprednisolone	15	18.7344 ± 2.7802	17.0261 ± 1.2711^{x}
EAFPA	125	25.0804 ± 5.8013^{y}	7.0026 ± 2.3496^{x,y,z}
EAFPA	250	21.2914 ± 5.5080^{y}	6.7844 ± 2.1493^{x,y,z}

Values are expressed as mean ± SD, (n=5), Comparison of control group with all groups. x = significant with control group (p<0,05), y = significant with levamisole group (p<0,05), z = significant with methylprednisolone group (p<0,05).

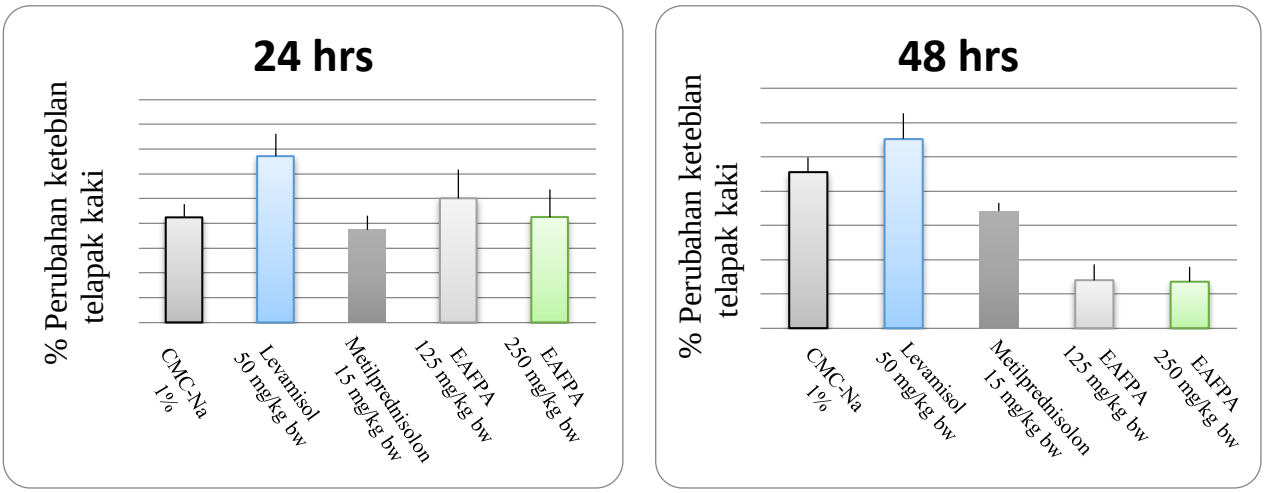

Fig. 1: Percentage of footpad thickness in 24 hrs and 48 hrs

Titer antibody response to SRBC

The reaction of an antibody and antigen can be easily detected by agglutination (clumping) of the antigen. If the antigen is an erythrocyte the term hemagglutination is used. Agglutination tests can also be used to measure the level of antibodies to particulate antigens. In this test, serum containing antibodies was collected from animals of each group and serial dilutions were done in microtiter plate. Fixed number of SRBC (50 µl) were added into each well. The maximum serum dilution that shows visible agglutination was considered as antibody titer. Humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody screening plasma cells. Antibody functions as the effectors of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are readily ingested by phagocytic cells13,14(Table 2). While immunosuppressant group i.e. Methylprednisolone treated group (15 mg/kg bw i.p), showed significant inhibition of haemagglutination titre (5.0939 ± 0.5037) in primer and (5.8164 ± 0.4257) in secondary as compared to control group (6.298 ± 0.5037) in primer and (6.9001 ± 0.5037) in secondary. Immunostimulation of humoral response by standard immunomodulatory drug Levamisole has resulted in higher antibody titre (p<0.05) as compared to control group. Mild potentiation of humoral immunity was observed in all EAFPA groups, while decrease humoral immunity was observed in treated animals (p<0.05). Dose dependant increase in titer antibody value was observed only with methanolic extracts.

Table 2.EAFPA effect on titerantibody response using SRBC as an antigen in mice

Treatment	Dose (mg/kg bw)	Titer antibody	
		Primer	Secondary
Control	-	6.298 ± 0.5037^{z}	6.9001 ± 0.5037^{z}
Levamisole	50	6.9001 ± 0.5037^{x}	7.5022 ± 0.5037^{x}
Methylprednisolone	15	5.0939 ± 0.5037^{x}	5.8164 ± 0.4257^{x}
EAFPA	125	$5.0939 \pm 0.5037^{x,y}$	$5.9368 \pm 0.5037^{x,y}$
EAFPA	250	$5.3347 \pm 0.5037^{x,y}$	$6.1776 \pm 0.6864^{x,y}$

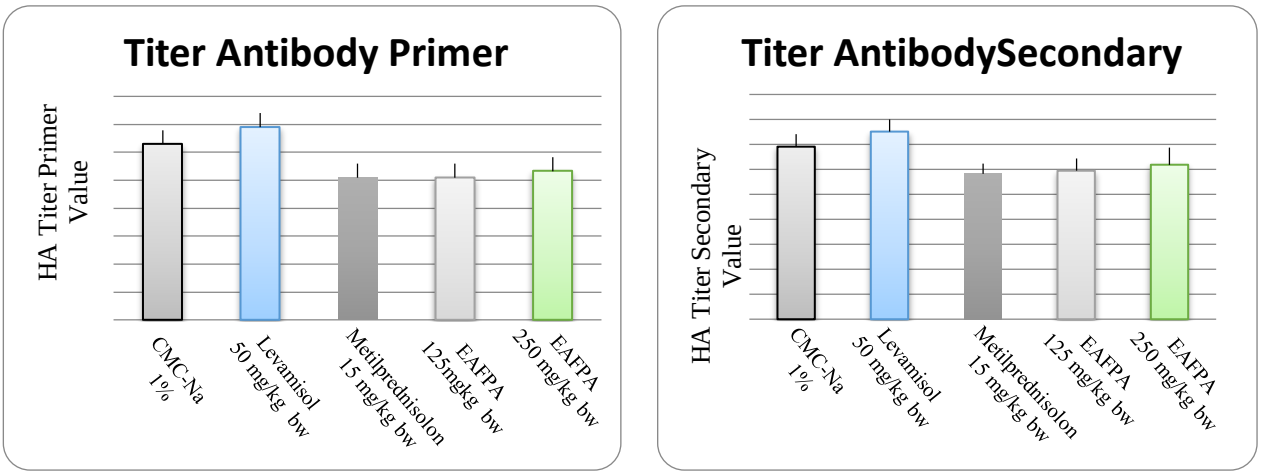

Fig. 2: Primer and secondary titre antibody in mice

Carbon Clearance Assay

In carbon clearence assay, ethyl acetat fraction of *Impatiens balsamina* Linn. were treated with all group showed concetration dependent phagocytic activity when compared to control group.The results of both parameters obtained can be seen in table 2 and 3.

Table 2. Phagocytic index and the cleaence rate of carbon from the circulating blood of mice

Treatment	Dose (mg/kg bw)	Rate Elimination (K_{el})	Phagocytic Index (k)	Classification
Control	-	-3.746	1.00	-
Levamisole	50	-4.804	1.28	Immunostimulant
Methylprednisolone	15	-2.825	0.75	Immunosupressant
EAFPA	125	-3.206	0.86	Immunosupressant
EAFPA	250	-3.880	0.74	Immunosupressant

Table 2 shows that the rate of carbon elimination in the methylprednisolone group with phagocytic index value was 0.75, it was lower than the normal control group, while the rate of elimination levamisole was higher than the normal control group with phagocytic index value was 1.28. It was showed that methylprednisolone had immunosuppressive activity and levamisole as an immunostimulant could be used as a positive control in the test non-specific immune response to treatment group.The test results were showed EAFPAdose 125 mg/kg bw and 250 mg/kg bw had a carbon elimination rate was lower than the normal control group. EAFPA125 mg/kg bw and 250 mg/kg bwhad phagocytic index value was lower than the normal control group ($k < 1$), were 0.86 and 0.74 respectively. It can be indicated ethyl acetate fraction of *Impatiens balsamina* Linn.had immunosuppressant effects on the cellular immune response in non-specific immune system.

Organ Index

Percent organ indexes of each group can be see in table 3.

Treatment	Dose (mg/kg bw)	Organ index (%)	
		Liver	Spleen
Control	-	4.789 ± 0.380	0.463 ± 0.031
Levamisole	50	5.075 ± 0.457	0.506 ± 0.065
Methylprednisolone	15	4.447 ± 0.418	0.365 ± 0.055*
EAFPA	125	4.579 ± 0.311	0.384 ± 0.055*
EAFPA	250	4.395 ± 0.216	0.347 ± 0.013*

Statistical analysis: * = ($p<0,05$) sig differences to control

Methylprednisolone and EAFPA 125 mg/kg bw and 250 mg/kg bwwere showed a decrease spleen index compared to normal control group. The decline in organ index also were showed significant difference ($p<0.05$) compared to the control and it was showed a decrease in the immune response, particularly the nonspecific innate immune response.Exposure spleen by a foreign substance was increased the activity of the spleen. When the spleen immune activity increased, the size and activity of lymphocyte proliferation also were increased so that the morphology of spleen size becomes larger . However, the treatment of EAFPAwasdeclined spleen index becomes smaller. It was believed to be the role of naftakuinon compounds had immunosuppressive effect that can inhibitted NF- kB as transcription factor of expression of immune response.

Conclusion

EAFPA at doses of 125 and 250 mg/kg bw has immunosuppressant effect with an effective dose of 125 mg/kg bw. EAFPA revealed the presence of the following classes of chemical compounds: polyphenols, flavonoid, steroid/triterpenoidandkuinons. These constituents are well established for their anticancer, antiproliferative, chemopreventive,chemotherapeutic. EAFPA supress both cellular and humoral immune systems.Immunomosuppresant potential of EAFPAcould be attributed for the presence of naphthoquinone which may modulate one of the above mentioned immune-mechanisms.

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