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Evaluation of Ethanolic Extracts of Mullaca (*Physalis angulata* L.) Herbs for Treatment of Lupus Disease in Mice Induced Pristane

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Abstract

Systemic lupus erythematosus is a systemic autoimmune disease characterized by multisystem inflammation due to immune complex deposits in the organs like kidneys, joints, pleura, skin, and so on. *Physalisangulata* known as mullaca, has been widely studied for its pharmacological activities such as antiinflammatory, immunosuppressive, cytotoxic, and also inhibition of organ rejection in transplantation. This study was directed to investigate the activities of *P. angulata* extract as an immunomodulatory agent. *P. angulata* powder was extracted by maceration with 70% of ethanol. An animal model of lupus was obtained by an injection 0.7 mL of pristane, i.p. Successful induction obtained in two weeks after injection which can be monitored by measuring total leukocyte count. For ensuring successful induction, another test was done four weeks and eight weeks after injection by detecting the presence of specific antinuclear antibodies using SDS PAGE method. Other measured parameters were including nonspecific immune response (measurement of total leukocyte count and differential leukocyte count), specific humoral immune response (hemagglutination test), specific cellular immune responses (delayed type hypersensitivity test), organ index and histology of kidney and spleen. As the results shown, ethanolic extract of *Physalisangulata* at dose of 1000 mg/kg BW orally, gave immunomodulatory effect in Lupus rat model. The extract worked primarily on specific immune response by lowering immune response near to the normal value, it was not suppressing immune response as prednisone.

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*Keywords:*lupus; mullaca; *Physalis angulata*; pristane; prednisone; imunomodulator

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1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease that is generally attacks women in age of 20 to 40 years with the prevalence ratio between women and men about 10: 1, respectively. SLE strike 1 of 700 women in productive age (20 to 40 years)[1]. Aetiology of SLE was not yet known clearly. SLE allegedly arises as a result of the interaction of genetic factors, environment, and hormonal that causes an immune response become reactive so they could not distinguish between self and nonself. Most of the sufferers are in productive age. In Indonesia, the number of sufferers of the lupus disease is not known precisely but it is estimated around 1.5 million of people (Ministry of Health of Indonesia).

The main problem in lupus treatment comes from the difficulties in diagnoses. Early lupus diagnosis were hard to be proven as the early symptom is not specific, resembling various other diseases. As the lupus progress, the treatment becomes harder. Currently, treatments for lupus still directed for decreasing the symptomatic events while the cause of the disease still outreach of the treatment. Another problem is the expensive price of drugs for therapy lupus.

This study was directed for searching alternative treatment of Lupus that can be affordable and effectively act on the cause of the disease. *Physalis angulata* has been proven active in immune systems by its supressing T cell proliferation, lymphocyte function, and macrophages activation effect[1–3]. This study was conducted to determine the immunomodulatory effects of Mullaca herbs in Lupus animal model which was induced by Pristane. The effect of imunomodulator was determined by measuring the specific and nonspecific immune responses after trials. As the immunomodulatory of mullaca could be proven, this herb will be useful as for lupus treatment.

2. Experiments

2.1. Materials

Crude drug of *Physalis angulata* L. aquadest, ethanol 70 %, CMC Na, pristane from Sigma-Aldrich, prednisone, SDS PAGE Kit , sheep red blood cells suspension. Female BALB/c mice ages 7-8 weeks weight 15-20 grams, purchased from PT. Biofarma.

2.2. Method

2.2.1. Animals grouping

Animals test divided into four groups: normal, control, prednison, and *Physalis angulata* extract treated group.

2.2.2. Induction

Induction of immune system in test animals performed with an injection of 0.7 mL pristane intraperitonealy[4].

2.2.3. Drug and Extract Treatment

Prednison was given with a dose of 18.2 mg/kg BW peroral once daily for seven days after induction. Ethanolic extract of Mullaca was given with a dose of 1000 mg/kg orally, once daily for seven days.

2.2.4. Measurements Immune Respons

Observations of the successful induction were performed two weeks after induction by measuring the total number of leukocytes. For ensuring successful induction, another test was done four weeks and eight weeks after

injection by detecting the presence of specific antinuclear antibodies using SDS PAGE method. At the end of therapy, measurements of immune response were conducted. Immunomodulatory effects was measured according to four parameters: nonspecific immune response using total leukocytes count, differential leukocyte count, and organ index of kidney and spleen; specific humoral immune response with antibody titers; specific cellular immune response with delayed-type hypersensitivity test, and renal and spleen histological observations[5].

2.2.5. Nonspecific Immune Response Parameters

2.2.5.1. Measurement of Total Leukocytes Count

Blood samples were taken and dilute in turk reagent before counting total leukocyte. Total leukocyte was measured under microscope.

2.2.5.2. Measurements of Differential Leukocytes

Samples of blood were stained with Giemsa dye, then leukocytes were identified and differentiated into neutrophils, monocytes, lymphocytes, eosinophils, based on differences in cell size, granule, and forms the nucleus of cells.

2.2.5.3. Determination of Kidney and Spleen Index

On the day of twelve after the last treatments, mice were sacrificed. Kidney and spleen were isolated and weighed. Kidney and spleen index was expressed as the weight of both organs per 100 grams of body weight.

2.2.5.4. Humoral Specific Immune Response Parameters

Humoral specific immune response parameter was evaluated by measuring antibody titer using hemagglutination (HA) test. Animals were grouped into 4 groups, each contain of 6 animals. Each animal recieved daily treatment for seven days.On the third day of the treatments, animals were challenged with 10% of sheep red blood suspension at the dose of 0.1 mL/ 10 gram i.p. Blood samples were collected on day 7, followed with centrifugation to obtain the serum. Phosphate buffer pH 7.2 0.5 M was added with the same amount of the serum.Phosphate buffer pH 7.2 was added to the serum and followed with serial dilution in microwell. Subsequently, each well was given 25 µLsuspension of sheep red blood cells with a concentration of 2% and incubated for 24 hours at temperature of 37°C. Primary antibody titer could be detected as hemaglutinationoccured in the well. To determine secondary antibody titer, mice were re-challenged with sheep red blood cells 10% at a dose of 0.1 mL/10g intraperitoneally. Blood samples were collected on day 5 after second injection of sheep red blood cells. Secondary antibody titers measured by the same method with the primary antibody titer.

2.2.6. Specific Cellular Immune Response Parameters

2.2.6.1. Delayed Type Hypersensitivity Test

Animals received the same treatment procedure as conducted in evaluation humoral specific immune response parameter. To induce delayed-type hypersensitivity response, treated mice were injected with sheep red blood cells2% subcutaneously on the right feet. Foot volume of treated mice was measured before induction, 24 hours and 48 hours after induction using plethysmometer. The difference betweenvolume of the foot before and after injection expressed in mL, used as a measurement of delayed-type hypersensitivity response.

2.2.6.2. Kidney and Spleen Histology

Histology was conducted using Hematoxyllin-eosin staining.

3. Results and Discussion

Systemic lupus erythematosus is a systemic autoimmune disease characterized by multisystem inflammation due to immune complex deposits in the organs like kidneys, joints, pleura, skin, and so on[6]. *Physalis angulata* known as mullaca, has been widely studied for its pharmacological activities such as antiinflammatory, immunosuppressive, cytotoxic, and also inhibition of organ rejection in transplantation[7–10].

Test animals were divided into four groups which are the normal, control, extract, and standards. All groups, except normal group, were induced by an injection of 0.7 ml pristine i.p. Pristane was used to induce immune response that resembling lupus condition as well. Successful induction could be observed by measuring total leukocytes count at 2 week after induction. In addition, presence of antinuclear antibody observed by SDSPAGE on 4th and 8th weeks after induction ensured that lupus immune response has been achieved.

Table1.Measurements of Total Leukocytes Count

Group Test	Total Leukocytes Count (/mm^3)
Non induction	12800 ± 2840.75
Induction	21500 ± 1759.98*

Description: *: p<0.05 compared to non-induction group

Analysis using t-student test showed that the number of leukocytes in induction groups was significantly different compared to the non-induction group. The presence of anti-nuclear antibodies by SDSPAGE confirmed existence of immune response of lupus. Specific anti-nuclear antibodies for lupus have a molecular size of 102-105 kDa[4]. In control group which induced by pristane showed thickness of line of specific anti-nuclear antibodies for lupus, whereas in normal group did not.

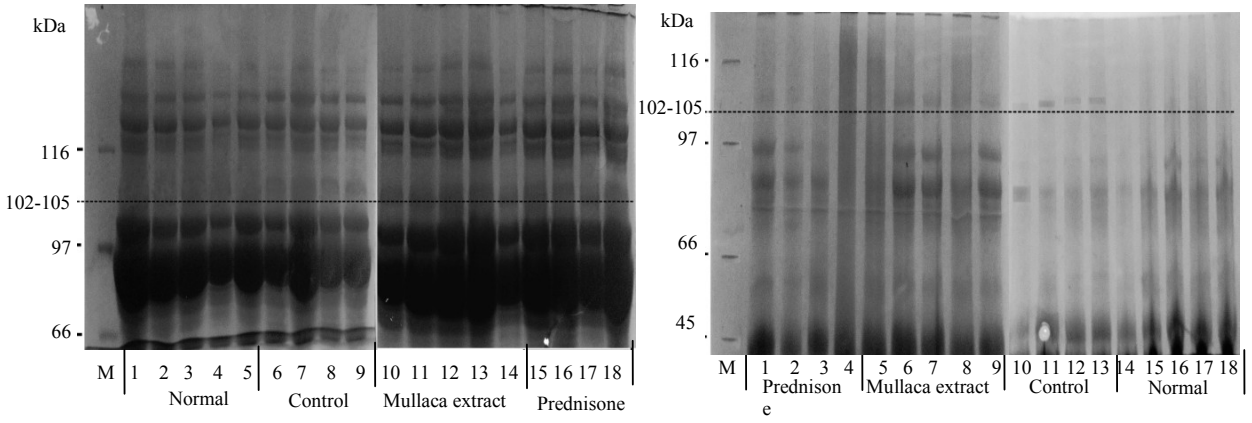

Fig. 1.Presence of antinuclear antibodies of lupus (102-105 kDa) confirmed by SDS PAGE of animal serum taken on 4th(a) M = Dna ladder, Line 1-5 = normal animal models, 6-9 = control animal models, 10-14 = Mullaca extract treatedanimal models, 15-18 = Prednisone treated animal models and 8th (b) week after induction, M = Dna ladder, Line 1-4 = normal animal models, 5-9 = control animal models, 10-13 = Mullaca extract treatedanimal models, 14-18 = Prednisone treated animal models

(b)

Table 2. Total Leukocytes Count in Blood of Mice after Extract Therapy

Group Test	Total Leukocytes Count (/mm^3)	Lymphocytes (/mm^3)	Eosinophils (/mm^3)
Normal	11600±596.33	60.26±453.688	985±242.69
Control	17500± 1490.06	7930±1454.92	4678±56.16
Extract	9217.97±924.99[a]	2950±270.14[a]	4124±247.69
Prednison	4000 ± 694.74[ab]	627±1181±488.819.87[a]	2381±488.81[a]

Description : a = p<0,05 compared to control, b = p<0,05 compared to normal group, dose of extract = 1000 mg/kg bw, Dose of prednisone = 18.2 mg/kg bw

Non-specific immune response was measured by total leukocytes and differential leukocyte count. Total leukocytes count is an important parameter of non-specific immune response. Increase in the number of total leukocyte could indicate the presence of an infection. Otherwise, it couldalso indicate existence of antigen detected by immune system.

Table 2 shows the difference of total leukocyte among the groups. The mice treated with extract had the same total leukocyte compared to normal groups but lower than control group. However it was still higher than of standard group treated with prednisone. Statistical analysis with one-way ANOVA showed significance different between extract group compared to prednisone and control group but not with normal group.

Differential leukocyte count revealed intriguing evidence. There were variations between groups in certain types of leukocyte.Lymphocyte number of groups treated with extract and prednisone was still higher than normal group yet considerably lower than control group. In addition, lymphocyte number of extract group is higher than prednisone group. Measurement of eosinophil also gave almost the same result. However, eosinophil number of extract group was still higher and not significantly different with control group.Looking to the result of total leukocyte number, total leukocyte number of extract group was closer to the normal group. Hence, it seems that ethanolic extract of mullaca could normalize the level of leukocyte after induction using pristane without suppressing another leukocyte component.

Another parameter that can also be used for assessing nonspecific immune response of immunomodulatory agent is index of certain organs involved in immune system, i.e. spleen and kidney. The spleen consists of red pulp and white pulp. There are free and fixed macrophages in the red pulp and macrophages also dendritic cells in high concentrations in white pulp. The larger organ index indicates the presence of stimulation toward immune system. Injection of pristane could overstimulate immune response reaching auto reactive level. Kidneys although not including the lymphoid organs were observed. Kidney organ index was observed due to the induction method of pristane would give the typical clinical manifestations of glomerulonephritis that indicated by an increase in kidney weight.

Table 3. Organ Index after Therapy with Extract

Test Group	Organ Index (%)	
	Spleen	Kidney
Normal	1.26 ± 0.06	1.23 ± 0.04
Control	2.02 ± 0.09	1.40 ± 0.04
Mullaca Extract	1.65 ± 0.11[a]	1.32 ± 0.03
Prednisone	1.65 ± 0.13[a]	1.33 ± 0.02

Description: a = p<0.05 compared to control group, n = 6 mice, dose extract = 1000 mg/kg bw, dose prednisone = 18.2 mg/kg bw

Table 3 shows organ index of all groups. Among the groups, organ index of normal group was the lowest. Control group showed the highest organ index caused by high proliferation of cells in spleen that eventually could indicate high response of immune system. Treatment with mullaca extract and prednisone considerably reduce the

organ index of spleen. It correlates with the effect of both prednisone and mullaca extract on decrease of total leukocyte number. There was not any considerable different in kidney organ index though kidney of lupus-induced mice had higher index. Slight decrease of kidney index found in both mullaca index and prednisone compared to control group. Analysis of the organ index was confirmed with spleen and kidney histology result.

Observations on spleen histology results in BALB / c mice with hematoxyllin - eosin staining using a light microscope at a magnification of 40X showed distribution of red pulp and white pulp of spleen varied in test animals. Control, mullaca extract, and prednisone groups showed an increase in white pulp area compared to normal group. White pulp contained macrophages and dendritic cells that play a role in the inflammatory response so that increasing in white pulp area indicated an increase in proliferation of macrophages and dendritic cells. Increased cell proliferation is a sign of an inflammatory reaction in the body of test animals. This was confirmed by the results of measurements of spleen index. Spleen index of control group, extract and prednisone increased compared to normal group. Extract group had lower spleen index and significantly different compared to control. This could be seen by visual observation of spleen histology. Extract group had extensive areas of white pulp which was smaller compared to control group, these suggested that extract had activity to decrease proliferation of immune cells in the spleen.

Observations kidney histology results in BALB / c mice with hematoxyllin - eosin staining using a light microscope at a magnification of 40X showed thickening of the glomerular membrane in control group when compared to normal group. While extract group had thickening of glomerular membrane that relatively small compared to the control groups. This suggests that extract had activity to reduce glomerulonephritis but it was not be able to restore condition of the kidney to normal state. In addition, from observation of kidney histology, it can be seen an increase in glomerular size in all groups. Increase in size was due to an increasing proliferation of cells in glomerulus. This result was consistent with the measurements of kidney organ index. Increase in kidney index in control, extract, and prednisone group compared to normal group, but this result was not significantly different between groups of animals. As there was no considerable different between testing group and also normal group in organ index and histology of kidney, glomerulonephritis was not occured at the time of the observation.

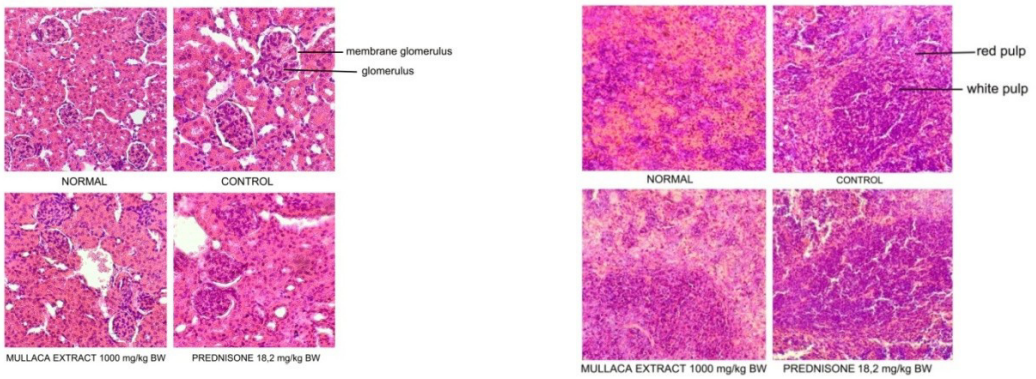

Fig. 2.Histology kidney and spleen of testing mice using hematoxyllin-eosin staining.

In addition to testing the effects on non-specific immune response, tests on specific immune response were conducted. Testing includes determining primary and secondary antibody titers as humoral immune response and delayed type hypersensitivity reactions as cellular immune responses. Antibody titer is referred to the highest dilution of serum of test animals that showed more than 50% agglutination upon reacting with external antigen. Primary antibody titers indicates the production of antibodies on first reaction against antigens while secondary antibody titer shows the extent of antibodies produced on the second or more exposure of antigens.

Table 4. Total Antibody Titer Of Mice After Therapy with Extract

Test Group	Antibody Titer	
	Primary	Secondary
Normal	1 : 64	1 : 128
Control	1 : 128	1 : 256
Mullaca Extract	1 : 64	1 : 64
Prednisone	1 : 16	1 : 32

Description: n = 6 mice, dose extract = 1000 mg/kg bw, dose prednisone = 18.2 mg/kg bw

Tabel 4 shows total antibody titer of mice after treatment. Primary antibody titer of extract and prednisone treated group were lower than control group. Extract treated group somehow produce primary closer to normal group and consider higher than prednisone treated group. The decreased in primary antibody titer might be due to the decrease in the activity of Th cells that work on stimulating B cells to produce antibodies. At the secondary antibody titers, all groups showed an increase in antibody titers when compared with primary antibody titer, but not higher than the control group. However, extract group experienced no increase in secondary antibody titers, suggesting that the extract could not increase the production of a secondary antibody. In other words ceplukan extracts could not increase the activity of B cells memory in formation of antibodies. This activity was good for people with lupus who had auto reactive immune system. From the results of the primary and secondary antibody titers showed that extract has antibody maintaining effect. However, mullaca extract could inihibit the memory of B cells as the secondary antibody titer was not raised up in mullaca extract treated tgroup. The antibody titer maintaining effect could be beneficial for lupus treatment as it need antibody balancing mechanism. verexpression of autoreactive antibody could be detrimental, yet oversuprressing antibody could turn down the immune response and give chance for infection to occur.

Delayed-type hypersensitivity reactions was evaluated for observing cellular imune activity. This reaction involves Th cell activity. Whenever Th cell acivity increases, it could secret cytokines that make macrophages more active, thereby increasing inflammatory reaction. This type of hypersensivity could be modeled by swelling tests on mice feet.

Table 5. Edema Volume of Mice Feet in Delayed Type Hypersensitivity

Test Group	% Change Thickness of Feet		Difference (%)
	T24	T48	
Normal	28.33± 5.16	-22.68± 5.25	5.65±5.21
Control	36.27 ± 5.98	-23.25 ± 3.91	13.02±4.95
Mullaca Extract	19.85 ± 4.25	-17.53 ± 4.32	2.32±4.29
Prednisone	36.28± 19.45	-22.44 ± 4.07	12.84±11.76

Description : n=6 mice, p = 0.05, dose extract = 1000 mg/kg bw, dose prednisone = 18.2 mg/kg bw

Table 5 shows mice feet edema volume of all groups on 24 and 48 hours after induction. Among all groups, group treated with mullaca extract showed lowest difference of feet volume on 24 hours and 48 hourse after induction indicating less edema than other groups. This result could be due to suppression of Th cell activity which in turn inhibited the inflammation in feet. Prednisone group experienced a high increase in thickness foot even closer to control group. This might be caused by late work of prednisone. Prednisone antiinflammatory effect was obvious just after reaching 48 hours after induction.

4. Conclusion

Lupus had been successfully modeled using pristane induction, and presence of anti-nuclear antibodies had been also confirmed by SDS PAGE. All of immunological parameters had been proven that mullaca (*Physalisangulata* L.) extract at dose 1000mg/kg body weight could be an alternative treatment for Lupus. However, its clinical efficacy and safety still need to be proven. This could be the challenge for further research.

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