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The effects of ethanolic extract of *Phaleria macrocarpa* (Scheff.) Boerl leaf on macrophage phagocytic activity in diabetic rat model

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ABSTRACT

Diabetic patients suffer from inflammation and immune deficiency due to the decrease macrophage activity that lead to vulnerable to infection. *Phaleria macrocarpa* (Scheff.) Boerl leaf extract has been proven increase macrophage activity and to have antiinflammatory effect. This study was conducted to investigate effect of ethanolic extract of *P. macrocarpa* (Scheff.) Boerl leaf (EEPML) on macrophage phagocytic activity in diabetic rat model. In addition, the M1 and M2 macrophage ratio was also evaluated. This was quasi experimental study with post test only control group design. Forty five male Sprague Dawley rats aged eight weeks were grouped into non diabetic control group, diabetic control group and diabetic treatment group which given with 7; 14; 28 mg/200 g body weight (BW) of EEPML respectively, orally once a day for 3, 14 or 25 days. Diabetic rats were induced by intraperitoneal injection of streptozotocin (STZ) at a dose of 65 mg/kg BW and nicotimamide at 100 mg/kg BW. Peritoneal fluid was isolated on day 3, 14 and 25 and cultured for the assay of macrophage phagocytic activity with latex beads. M1 and M2 macrophage percentages were analyzed by flowcytometry with anti CD40 and CD206 antibody. The results showed that the mean of active macrophages and macrophages phagocytic index of EEPML treatment groups on day 3, 14 and 25 were significantly higher than those in the diabetic control group ($p < 0.05$). Moreover, the mean of M1 macrophage percentage of EEPML treatment groups were significantly higher on day 14 but significantly lower on day 25 than that in the diabetic control group ($p < 0.05$). In addition, the mean of M2 macrophage percentage was not significantly difference among the groups ($p > 0.05$). In conclusion, the ethanolic extract of *P. macrocarpa* (Scheff.) Boerl leaf administration can increase macrophage phagocytic activity in diabetic rats. In addition, it also can increase M1 macrophage percentage on .day 14

ABSTRAK

Penderita diabetes mengalami inflamasi dan penurunan sistem imun akibat menurunnya aktivitas sehingga rentan terhadap infeksi. Ekstrak daun mahkota dewa (*Phaleria macrocarpa* (Scheff.) Boerl) telah dibuktikan dapat meningkatkan aktivitas makrofag dan mempunyai efek antiinflamasi. Penelitian ini bertujuan untuk mengkaji efek ekstrak etanol daun mahkota dewa (EEMD) terhadap aktivitas fagositosis makrofag pada tikus model diabetes. Selain itu rasio makrofag M1 dan M2 juga dihitung. Penelitian ini adalah

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penelitian eksperimental semu dengan rancangan uji akhir dan kelompok control. Empat puluh lima tikus jantan Sprague Daley berumur delapan minggu dikelompokkan menjadi kelompok kontrol non diabetes, kelompok kontrol diabetes dan kelompok perlakuan EEMD dosis 7, 14 dan 28 mg/kg berat badan secara oral sekali sehari selama 3, 14 atau 25 hari. Tikus dibuat diabetes dengan diinduksi streptozotisin dosis 65 mg/kg berat badan dan nikotinamid 100 mg/kg berat badan secara intraperitoneal. Cairan peritoneal tikus diisolasi pada hari ke 3, 14 dan 25 dan dikultur untuk pengujian aktivitas fagositosis makrofag terhadap partikel lateks. Persentase makrofag M1 dan M2 dianalisis dengan flositometri menggunakan anti CD40 and antibody CD206. Hasil penelitian menunjukkan rerata makrofag aktif dan indeks fagositosis makrofag kelompok perlakuan EEMD pada hari ke 3, 14 dan 25 lebih tinggi secara bermakna dibandingkan dengan kontrol diabetes ($p < 0.05$). Selanjutnya, rerata persentase makrofag M1 kelompok perlakuan EEDM pada hari ke 14 lebih tinggi secara bermakna dibandingkan dengan kontrol diabetes ($p < 0.05$), akan tetapi lebih rendah secara bermakna pada hari ke 25 ($p < 0.05$). Sedangkan persentase makrofag M2 tidak berbeda nyata diantara kelompok uji ($p > 0.05$). Dapat disimpulkan, pemberian ekstrak etanol daun mahkota dewa dapat meningkatkan aktivitas makrofag pada tikus diabetes. Selain itu, pemberian ini juga dapat meningkatkan persentase makrofag M1 pada hari ke 14.

Keywords: diabetes mellitus - peritoneal macrophage – phagocytosis - M1 macrophage - M2 macrophage

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder of endocrine characterized by hyperglycemia as the result of insulin deficiency, decrease in tissue insulin sensitivity or both.¹ The prevalence of DM worldwide increased dramatically in the last 20 years. The World Health Organization (WHO) estimated that the people with DM will increased to be 300 million in 2025 in the world.² Indonesia is the fourth country with the highest people with DM in the world after India, China and America. In Indonesia, the people with DM reached 12.2 million in 2013, it will be 21.3 million in 2030.³ The pathogenesis of DM is associated with the inflammatory process which oxidative stress and chronic inflammation can induce insulin resistance. Diabetes mellitus is also the manifestation of inflammatory response such as migration and macrophage infiltration into the islets of Langerhans of pancreas, the increase in C-reactive protein, fibrinogen, pro-inflammatory cytokines (IL-6 and TNF α),

and the decrease in IL-10. Oxidative stress in diabetes also increases the death of cells and apoptosis.⁴ Macrophage is phagocytic cells in innate immunity system that plays an important role in inflammatory process and body immunity system. It consists of two sub-populations, M1 and M2 macrophages. M1 macrophages play their role in the inflammatory stage to produce pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, IL-12, IL-18 and IL-23. They are transformed into M2 macrophages in the resolution phase of inflammation. M2 macrophages produce anti-inflammatory cytokines such as IL-10, TGF β , IL-1Ra and IL-18BP.⁵

Macrophages play their role in inflammation and infection in diabetic case. Activation of M1 macrophages induces inflammation and insulin resistance. M2 macrophage activation can forestall pancreatic β cell auto antigen in type I diabetes mellitus and inhibit the development of type II diabetes.^{6,7} Dysfunction of innate immunity system and the decrease in phagocytic

activity of macrophages can increase the risk for infection in DM.⁸ *Phaleria macrocarpa* (Scheff.) Boerl, locally well known as *mahkota dewa*, is an endogenous medicinal plant from Papua, Indonesia that widely used by Indonesian and Malaysian people. This plant is traditionally used to treat DM, allergy, liver diseases, vascular diseases, cancer, kidney failure, stroke, and hypertension. The parts of the plant used as medicine are stems, leaves, and fruit.⁹ The toxicity and mutagenicity of the *P. macrocarpa* (Scheff.) Boerl. fruit have been evaluated and showed that this fruit is safe for use as medicine.^{10,11}

Phaleria macrocarpa (Scheff.) Boerl. leaf contains the active compounds including flavonoids, polyphenols, saponins, tannin, and steroids which have anti-microbial effects.^{12,13} Phalerin is a specifically active compound found in its leaf as an anti-inflammatory agent.^{14,15} The extract of its leaf has been reported to have antihyperglycemic effects through the inhibition of α -glucosidase activity which is a carbohydrate-digesting enzymes.¹⁶ This plant also has antioxidant activity, tyrosinase inhibition, and analgesic effect.^{17,18} Ethanolic extract of *P. macrocarpa* (Scheff.) Boerl. leaf can increase splenic NK1.1 cells activity and macrophage phagocytic activity in mice.¹⁹ In this study we reported the effect of ethanolic extract of *P. macrocarpa* (Scheff.) Boerl leaf (EEPML) on macrophage phagocytic activity in diabetic rat model. Moreover, the M1 and M2 macrophage ratio was also determined.

MATERIALS AND METHODS

Extract preparation

Phaleria macrocarpa (Scheff.) Boerl leaf was collected from Bantul District, Yogyakarta and identified in Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta,

then dried and powdered. Powdered leaves were macerated with 70% ethanol at room temperature for 24 h and then filtered to separate filtrate from residue. The residue was remacerated three time and filtrates obtained were collected and dried using vacuum rotary evaporator to obtain dried extract. Extract preparation for testing was prepared in 5% polyethylene glycol (PEG) solution.

Diabetes animal model

Forty five male Sprague Dawley rats aged eight weeks with the body weight (BW) of 200 to 230 g obtained from the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta were used in this study. The rats were housed at room temperature under 12 hours cycles of dark and light and fed a standard food as well as provided an access to aquadest *ad libitum*. After an acclimatization period of one week, the rats were then grouped into non diabetic or normal control group, diabetic control group and diabetic treatment group which given with 7; 14; 28 mg/200 g BW of EEPML respectively, orally once a day for 3, 14 or 25 days according to their terminating groups. Diabetic rats were induced by single intraperitoneal injection of streptozotocin (STZ) at a dose of 65 mg/kg BW and nicotinamide at 100 mg/kg BW. Fasting blood glucose level was examined on day 8 after STZ induction. Diabetic rats were diagnosed if blood glucose level was >170 mg/dL. Protocol of the study was approved by the Ethical Commission for Preclinical Research of the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta.

Isolation and culture of peritoneal macrophages

The rats were sacrificed on day 3, 14 or 25 according to their terminating groups. The

rats were anaesthetized by xylazine ketamine injection intramuscularly on their left thighs and cervical dislocation was performed on supine position. The abdomen skin was opened and the peritoneum sheath was cleaned up with 70% of ethanol. Approximately 10 mL of cold RPMI medium were injected into the peritoneal cavity during 3 min while they were shaken slowly. Peritoneal fluid was excreted from peritoneal cavity by pressing the organ with two fingers. Aspirated fluid using injecting tube was conducted on non-fat part and which was far from intestine. Aspirated centrifugation was at 1,200 rpm at the temperature of 4°C in 10 min. The established supernatant was removed and 3 mL of complete RPMI medium on the pellet was added. The number of cells was counted with hemocytometer and their viability was determined by using trypan blue solution. The solution was re-suspended with complete RPMI medium so that cell suspension with the density of 2.5×10^6 cells/mL was obtained. The calculated cell suspensions were inoculated in a 24-well plate, equipped with cover-slip with each well contained 200 mL suspension (5×10^5 cells). The cells were incubated in a 5% CO₂ incubator at the temperature of 37°C in 30 min, and 1 mL of complete RPMI medium was added in each well, followed by incubation within 2 h. The cells were washed with RPMI-1640 twice and 1 mL of complete RPMI medium was added in each well, followed by incubation within 24 h.

Macrophage phagocytic activity test

Latex beads were resuspended in PBS with 2.5×10^7 /mL concentration. Peritoneal macrophages that had been cultured in the previous day were washed twice with RPMI-1640. Suspension of latex beads (200 mL/well) and the sample (200 mL/well) were added to each well and then were incubated in

5% CO₂ incubator at the temperature of 37°C in 60 min. Following after the incubation, cells were washed with PBS three times to remove the excess of latex beads, dried at a room temperature, and fixed with absolute methanol in 30 sec. The cells attached to cover slips were tinged with 20% of Giemsa in 20 min then washed with distilled water. The percentage of 100 examined macrophage cells that phagocyte latex beads and the number of latex beads phagocytized by macrophages were counted by using a light microscope with 400x magnification.

Flowcytometry analysis

Peritoneal macrophage cell suspension which contained approximately 5×10^5 cells was added with anti-CD40 antibody FITC (Ebioscience 11-0402-82) and anti-CD206 antibody/MRC1 (BIOSs bs-4727R-Cy5.5) and incubated in 30 min at the temperature of 4°C. The cells were then washed twice, resuspended into pharmigen stain buffer, examined by using flowcytometer FACS Calibur, and analyzed by using Cell Quest software. The data obtained from the flowcytometry analysis constituted the percentage of M1 macrophages and M2 macrophages.

Statistical analysis

Data of the percentage of active macrophages (AM), phagocytosis index (PI), and the percentage of M1 and M2 macrophages were presented as mean \pm standard deviation (SD). Normality of data distribution was tested by using the Shapiro-Wilk. One way analysis of variance (Anova) and Tukey post hoc tests were used for normal distributed data. Non-parametric Kruskal Wallis and Mann Whitney post hoc tests were used for abnormal distributed data.

RESULTS

The morphology of macrophages in normal control group and in the EEPML treatment groups were bigger with protruded

cytoplasm and phagocytosed many latex beads, while macrophages in diabetes control group were smaller and rounded and phagocytosed fewer latex beads (FIGURE 1).

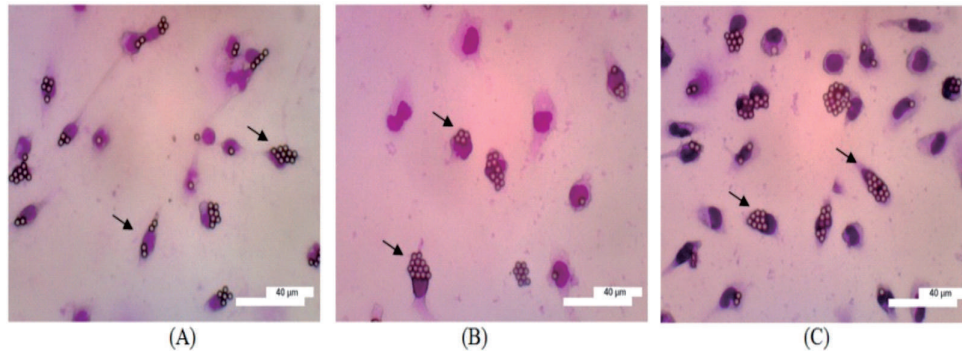


FIGURE 1. Macrophage phagocytic assay of normal control group (A) and diabetic treatment group (C) showed more active phagocyte latex beads (arrow) than diabetic control group (B). Giemsa staining, 400x magnification, scale 1:400µm.

The mean of active macrophages (AM), phagocytic index (PI), M1 and M2

macrophages of all groups on day 3, 14 and 25 are presented in TABLE 1.

TABLE 1. The mean of active macrophages (AM), phagocytic index (PI), M1 and M2 macrophages of all groups on day 3, 14 and 25

Groups		Parameter			
Termination	Treatment	Active Macrophage (%)	Phagocytic Index	M1 Macrophage (%)	M2 Macrophage (%)
Day 3	N	88.3±0.6	526.7±25.7	45.7±7.9	64.5±8.6
	DM	77.3±1.2	323.3±30.9	28.8±7.8	57.4±17.6
	EEPML1	81.3±1.2	350.0±20.4	23.5±15.4	52.0±23.0
	EEPML2	78.7±2.1	369.3±33.8	38.9±4.6	70.2±13.1
	EEPML3	82.7±2.3	407.7±62.6	38.6±10.6	62.0±11.3
Day 14	N	87.3±2.3	473.3±59.8	50.7±20.5	60.1±26.2
	DM	65.0±2.7	275.3±59.3	48.1±24.3	59.1±28.0
	EEPML1	72.0±3.6	325.0±44.0	60.7±10.0	77.1±0.7
	EEPML2	87.3±5.0	383.0 ±56.7	69.4±7.1	76.1±2.3
	EEPML3	83.0±5.6	321.3±30.0	51.9±6.2	72.4±2.0
Day 25	N	92.0±3.5	525.7±98.3	51.2±2.2	74.8±3.4
	DM	76.3±6.0	259.7±30.1	45.1±6.1	75.0±4.4
	EEPML1	90.0±1.0	384.7±19.6	46.5±4.9	74.7±9.1
	EEPML2	90.7±3.1	341.0± 68.6	24.7±6.1	74.3±0.6
	EEPML3	84.7±1.5	335.0±87.7	46.4±16.0	74.1±1.6
p		0.000**	0.000*	0.002*	0.165**

Normality test with Saphiro-Wilk, performed by the mean value of ± SD. N: Normal control group; DM: diabetic control group; EEPML: diabetic treatment group at doses 7, 14 and 28 mg/200 g BW.*One way Anova, p<0.05 significant, **Non parametric Kruskal Wallis, p<0.05 significant.

The mean of AM of the normal control group was significantly higher than that of the diabetic control group on all day of examination and than that of the diabetic treatment groups at all doses on day 3 ($p < 0.05$). However, it was not significantly different compared to that of the diabetic treatment groups at doses of 14 and 28 mg/200g BW on day 7 and at all doses on day 14 ($p > 0.05$). The means of AM of the diabetic treatment groups at all doses on day 3 and at dose of 7 mg/200g BW on day 7 were not significantly different compared to those of the diabetic control group ($p > 0.05$). However, at doses of 14 and 28 mg/200g BW on day 7 and at all doses on day 14, they were

significantly higher than those of diabetic control group ($p < 0.05$). The highest of the mean of AM in diabetic treatment groups was observed at dose of 14 mg/200g BW on day 25 (FIGURE 2.A).

The mean of PI of the normal control group was significantly higher than that of the diabetic control group on all day of examination and than that of the diabetic treatment groups at all doses on all day of examination ($p < 0.05$). In addition, the means of PI of the diabetic treatment groups at all doses on all day of examination were significantly higher than that of the diabetic control group ($p < 0.05$) (FIGURE 2.B).

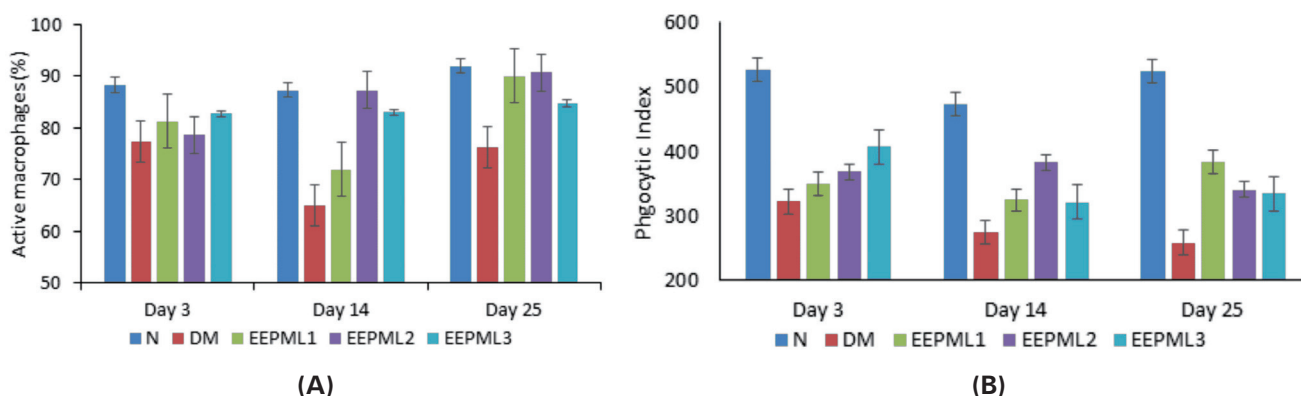


FIGURE 2. A) The mean of active macrophage (AM) and B) phagocytic index (PI) on day 3, 14 and 25; N: Normal control group; DM: diabetic control group; EEPML: diabetic treatment groups at doses of 7, 14 and 28 mg/200 g BW.

The mean of M1 macrophage percentage of the normal control group on day 3 was significantly higher than that of the diabetic control group on day 3 ($p < 0.05$). However, it was not significantly different compared to that the diabetic control group on day 7 and 14 ($p > 0.05$). The means of M1 macrophage percentage of the diabetic treatment groups at doses of 14 and 28 mg/200g BW on day 3 and at doses of 7 and 14 mg/200g BE on day 14 were significantly higher than that

the diabetic control group on day 3 and 7, respectively ($p < 0.05$). However, the means of M1 macrophage percentage of all groups on day 25 were not significantly different ($p > 0.05$), except the mean of M1 macrophage percentage at dose of 14 mg/200g BW which was significantly lower than others groups ($p < 0.05$) (FIGURE 3.A). Furthermore, the means of M2 macrophage percentage of all groups on all day of examination were not significantly different ($p > 0.05$) (FIGURE 3.B).

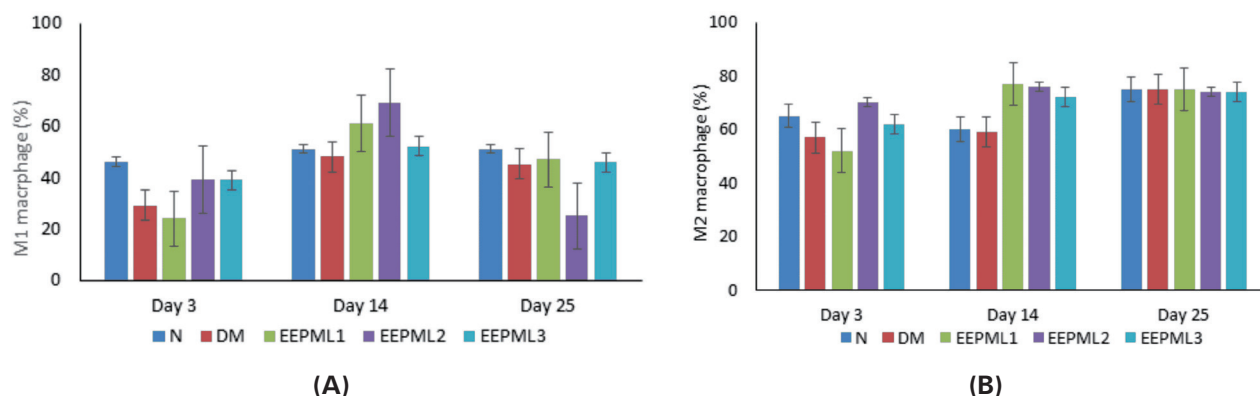


FIGURE 3. A) The mean value of M1 macrophage and B) M2 macrophage percentage on day 3, 14, and 25. *post hoc Tukey (p<0,05). N: Normal control group; DM: diabetic control group; EEPML: diabetic treatment groups at doses of 7, 14 and 28 mg/200 g BW.

DISCUSSION

Diabetes mellitus is associated with the innate immune system disturbance that caused patients with DM exhibit increased susceptibility to infection, impaired wound healing and other inflammatory or degenerative manifestations. Macrophages play important roles in controlling innate immune system.^{8,20} In this study, the decrease in macrophages activity was observed in diabetic rats as indicated by lower of the mean of AM and PI compared to normal rats on day 3 and continued on day 14 and 25 (TABLE 1 and FIGURE 2 and 3).

The decrease in the macrophages activity may be caused by hyperglycemia condition in DM which leads to oxidative stress and glycation induced the cells death. Hyperglycemia also increases advanced glycation end product (AGE) formation that will be bound to the AGE receptor (RAGE) on the membrane of various cells, including macrophages. The bond of AGE-RAGE with macrophage cells can inhibit the macrophages phagocytic activity.²¹ In addition, inflammatory condition in DM can also induce the formation of inflammatory mediator of high mobility group box 1 (HGMB1) which can bind to

phosphatidylserine on the membrane of apoptotic cells. This condition can inhibit the macrophage capacity to identify apoptotic cells and to perform efferocytosis on the apoptotic cells lead to inhibition of tissue repair.²²

The EEPML is potential to increase the peritoneal macrophage phagocytic activity and improve the function of immunity system in diabetic condition. The three doses of EEPML have similar effects to increase macrophages activity. However, the most effective dose of EEPML was 14 mg/200g BW. This dose EEPML could increase the macrophages activity similar to that normal control group on day 3, 14 and 25 (TABLE 1 and FIGURE 2 and 3). The immunomodulatory effect of *P. macrocarpa* (Scheff.) Boerl. leaf extract has been reported in previous studies. Ghufron *et al.*¹⁹ reported that ethanolic extract of *P. macrocarpa* (Scheff.) Boerl. leaf can increase splenic NK1.1 cells activity and macrophage phagocytic activity in mice. Abood *et al.*²⁰ reported an immunomodulatory effect of *P. macrocarpa* (Scheff.) Boerl. and their isolated fraction by increasing macrophage cell proliferation and significant induce of cytokines INF- γ , IL-6 and IL-8 intracellular expression.

Macrophages play an important role in the inflammatory process in diabetic condition. This study showed the percentage of peritoneal M1 macrophages in diabetic control group significantly decreased on day 3 compared to normal control group, while the percentage of peritoneal M2 macrophages was unchanged (FIGURE 3A and B). The decrease of the peritoneal M1 macrophage percentage was caused by the decrease in the macrophages phagocytic activity due to the immunity system dysfunction in diabetic condition. M1 macrophages are pro-inflammatory macrophages that play an active role in performing phagocytosis while M2 macrophages are anti-inflammatory macrophages that actively perform effecytosis to clean up apoptotic cells for tissue repair.^{6,21} M2 macrophages are commonly accumulated in normal energy metabolism process involving mitochondria. Meanwhile, M1 macrophages are generally accumulated at edema, hypoxia, anaerobic glycolysis, inflammation, and insulin resistance condition.²²The EEPML administration influenced the percentage of peritoneal M1 and M2 macrophages in diabetic condition. It could increase the mean of the percentage of peritoneal M1 macrophages especially on day 14 (FIGURE 3.A). The increase of the percentage of peritoneal M1 macrophages can maximize inflammatory state of diabetes, together with the increase in the macrophage phagocytic activity induced by the EEPML administration. Moreover, it could also increase the mean of the percentage of peritoneal M2 macrophages on day 14, however it was not significantly different (FIGURE 3.B). The increase of the percentage of peritoneal M2 macrophages is caused by the increase of percentage of peritoneal M1 macrophages and its activity inducing the M1 macrophages polarization into M2 macrophages to relieve inflammatory conditions in diabetes.

The result of this study is different from the previous study that reported the topically EEPML ointment administration on diabetic wound on rats can increase the M1 and M2 macrophages on day 3, however it decrease on day 14.²³ The different may be caused by the macrophages used in these study. The inflammatory process in diabetic wound involves the tissue-resident macrophages distributed in the epidermis. In this study, we used peritoneal macrophages containing the combination of the tissue-resident macrophages and monocytes derived macrophages from the systemic circulation.^{24,25} The two types of macrophages might have the different time of activation.

CONCLUSION

In conclusion, the ethanolic extract of *P. macrocarpa* (Sheff.) Boerl leaf administration can increase macrophage phagocytic activity in diabetic rats. In addition, it also can increase M1 macrophage percentage on day 14. This immunomodulatory effect of the plant extract could be used to improve the immunity system of diabetic conditions.

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