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SYNERGETIC MODULATION OF T LYMPHOCYTE AND TER119$^+$ CELL USING COMBINATION OF *ELEPHANTOPUS SCABER* AND *POLYSCIAS OBTUSA* EXTRACT IN PREGNANT MICE AFTER *SALMONELLA TYPHI* INFECTION

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ABSTRACT

Pregnant women are more susceptible to infectious diseases because of the change of immune system. This study aimed to analyze the efficacy of *E. scaber* and *P. obtuse* which function as an immune-modulator in CD4$^+$ T cells, CD8$^+$ T cells, and TER-119$^+$ in pregnant condition after infected by *Salmonella typhi*. Pregnant mice were infected by *S. typhi* (1x10^7). Ethanolic extract of *E. scaber* and *P. obtusa* were given orally with formulation 100%:0%, 75%:25%, 50%:50%, and 25%:75% with an initial dose of 50 mg/kg BW. This study proved that *S. typhi* could decrease the number of CD4$^+$and CD8$^+$T cells, CD4$^+$CD62L$^-$ and CD8$^+$CD62L$^-$ T cells, and TER-119$^+$ cells. The formulation of *E. scaber* and *P. obtusa* gives a synergetic modulation for immune system activation by increasing the number of those cells, direct killing on *S. typhi*, and prevents immune cell death.

KEY WORDS: Erythrocyte, *E. scaber*, Lymphocyte, *P. obtusa*, Pregnant

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INTRODUCTION

Pregnant women have a unique body condition because in the pregnant condition, many physiological changes occur. First, pregnant women have higher risk and are more susceptible to infectious diseases. That is because they have a unique immunological condition due to their pregnancy. Immune adaptation must occur in pregnancy because it is very important for fetus survival while maintaining the mother's ability to fight infection. The immune system significantly changes during pregnancy and these changes were necessary to support normal placentation and pregnancy. Second, pregnant women are desperate in the need of the amount extras of iron because iron is needed for the development of plasma, placenta, and iron transfer to the fetus. Although there is a deficiency in mother's body, the iron will still be transferred to the fetus[1]. Based on that, pregnant women are very susceptible to iron deficiency and impact on the reduction of erythrocyte formation. TER-119 is used as a marker for cell erythroid pathway so the expression level of TER-119$^+$ can describe the development of mature erythrocyte. Actually TER-119$^+$ is needed in immune system. Leukocyte and erythrocyte work in synergy within the immune system to fight a pathogen in the body[2]. Although pregnant women are susceptible to infectious diseases, they are not encouraged to consume drugs freely. Bad influence of drugs on the fetus can be toxic, teratogenic or lethal, depending on the characteristic of drug and gestational age. Toxic influence exists when the drugs are taken during pregnancy and causes a physiological disorder or bio-chemistry of the fetus, and new symptoms usually appear shortly after birth. The influence of teratogenic drug causes anatomic malformations in fetal organ development. This drug's influence usually occurs at sub lethal doses. If the influence of drugs is lethal, it results in the death of a fetus. Therefore, women need a natural active compound which can enhance immune system to defense herself and her fetus from the infectious diseases during pregnancy. Indonesia is rich in biodiversity, including plants which can be used as raw materials for medical treatments[3]. Tapak liman (*Elephantopus scaber*) in Indonesia is one of them. *E. scaber* is one of traditional medicinal plant that contains *epiphrieelinol, lupeol, stigmasterol, triacontan-1-ol, dotriacontan-1-ol, lupeol acetate, deoxyelephantopin, isodeoxyelephantopin, lactone, flavonoids, polyphenols lutein-7* and *glucoside*. *E. scaber* has been used for traditional drugs in fresh, dry powder and encapsulated tablets. Several types of diseases that can be effectively addressed by using *E. scaber* include inflammatory diseases such as inflammation of the tonsils, influenza, throat, eyes, kidney, and vaginal discharge acute and chronic as well. *E. scaber* according to Marmi[4] giving positive impact for the immune system indicated by the increasing number of CD4$^+$ cells, CD8$^+$, TER-119$^+$, and B220$^+$. In normal circumstances, the immune system is mainly held by lymphocytes, which are carried out by two types of cells, B lymphocytes (B220$^+$) and T lymphocytes (CD4$^+$ and CD8$^+$ T cells)[5]. Another plant is Kedondong laut (*Poliscyas obtusa*) which has a potential role as an immune-modulator[6,7]. The targets of immune-modulatory compounds are macrophages, granulocytes, and lymphocytes T and B[8]. *P. obtusa* contains *Polysciosida A-H, flavonoids,* and *dexamethason*. Our recent study proved that crude extract of *P. obtusa* could stimulate of CD8+, CD4+, B220+, and regulatory T cells in broiler chicken infected by *Salmonella* for immunological self-tolerance[6,7]. Those results proved that this plant can be used to stimulate immune system. Thus, formulation of those two plants for herbal medicine will be giving systematical effect in female reproduction especially in activating immune system to eliminate infection from pathogen. The aim of this study was to investigate the role of formulation of *E. scaber* and *P. obtusa* to immune system especially in subset CD4$^+$ T cells, CD8$^+$ T cells, and TER-119$^+$ in pregnant condition after infected by *Salmonella typhi*.

MATERIALS AND METHODS

Experimental animal preparation

The experimental animal in this study was pathogen free females Balb/C mice whose age was 8 weeks old. After 7 days of acclimation, the preparation of insemination was done. The first day of pregnancy was determined by the presence of a vaginal plug. Positive pregnant mice had been separated and grouped into treatment groups. An intra-peritoneal injection of 10^7 of *Salmonella typhi* was given on day 7 of pregnancy.

Extraction process

The fresh and undamaged of *E. scaber* and *P. obtusa* leaves were selected, then plucked from the first leaves to the fifth from the shoots of plants. The leaves were washed thoroughly, and wind-dried and blended to make a powder. The powder was macerated in 96% ethanol for 3 days, and then filtered with a Buchner funnel and the filtrate was obtained. The filtrate was then concentrated by rotary evaporator at a maximum temperature of 60°C.

Oral Administration of E. scaber and P. obtusa

E. Scaber and *P. obtusa* leaf extract was given orally to mice for on the day 14 and 18 with initial dose 50 mg/kg BW, according to the treatment group. There were treatment groups of:

K1 = non-pregnant mice were neither infected by *S. typhi* nor given the extract
K2 = pregnant mice were neither infected by *S. typhi* nor given the extract
K3 = pregnant mice were both infected by *S. typhi* and given the extract
P1 = 100% ES : 0% PO
P2 = 75% ES : 25% PO
P3 = 50% ES : 50% PO
P4 = 25% ES : 75% PO

Experimental Animal Dissection and Cell Isolation

Dissection was on the day 14 (starter) of pregnancy and on the day 18 (finisher) of pregnancy. Spleen and bone marrow were isolated and washed with PBS in a petri dish. Cells were isolated from spleen by crushing them in PBS. Cells in bone marrow were isolated from femoral bone. Homogenates were centrifuged at a speed of 2500 rpm, at a temperature of 10° C, for 5

minutes. Supernatant was discarded while the pellet was resuspended in 1 ml of PBS.

FACS Analysis

100 µl of spleen cell suspensions were put in microtube A whereas bone marrow cell suspensions put into microtube B was 200 µl. Both A and B microtubes were centrifuged with the speed of 2500 rpm for 5 minutes at the temperature of 10°C. Supernatant was discarded and the pellets were stained with antibodies. The 2 types of dye combination were used, namely the antibody composition as follows: dye A: FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD8, and PE/Cy5-conjugated rat anti-mouse CD62L; dye B: PE/Cy5-conjugated rat anti-mouse TER119. Cells were stained with antibodies and then incubated for 20 minutes in the ice box at 10° C. The incubated cells were added with 500 µl of PBS. Each sample was transferred into a flowcytometry cuvette and then analyzed by flowcytometer.

Statistical Analysis

The statistical analysis used a parametric one-way ANOVA analysis with the significance of 0.05% and was followed by Tukey test. All results are presented as the mean ± standard deviation values of 5 mice in each group. The application for statistical analysis was SPSS version 16 for Windows.

RESULTS

CD4+ and CD8+ T cells are cells which play important role in a cellular immune system. As shown both in Figure 1 and 2, the number of CD4+ T cells was reduced after 18 days of infection whereas CD8+ T cells was already reduced after 14 days of infection. It was indicated that the immune system of the treated animal was becoming weaker against infection. In this study we demonstrated that P1 formulation of *E. scaber* and *P. obtusa* could increase the relative number of CD4+ T cells after 14 days of oral administration, P3 formulation could increase CD4+ T cells after 18 days of oral administration, and P4 formulation could increase the number of CD8+ T cells after 14 days of oral administration. CD62L are cell surface adhesion molecules from the selected molecules. They are important in the regulation of immune system. They serves to pass adhesion and roll on endothelial cells along the blood vessels and mediate naïve T cells towards the peripheral lymphoid organs which are the sites of immune response initiation.

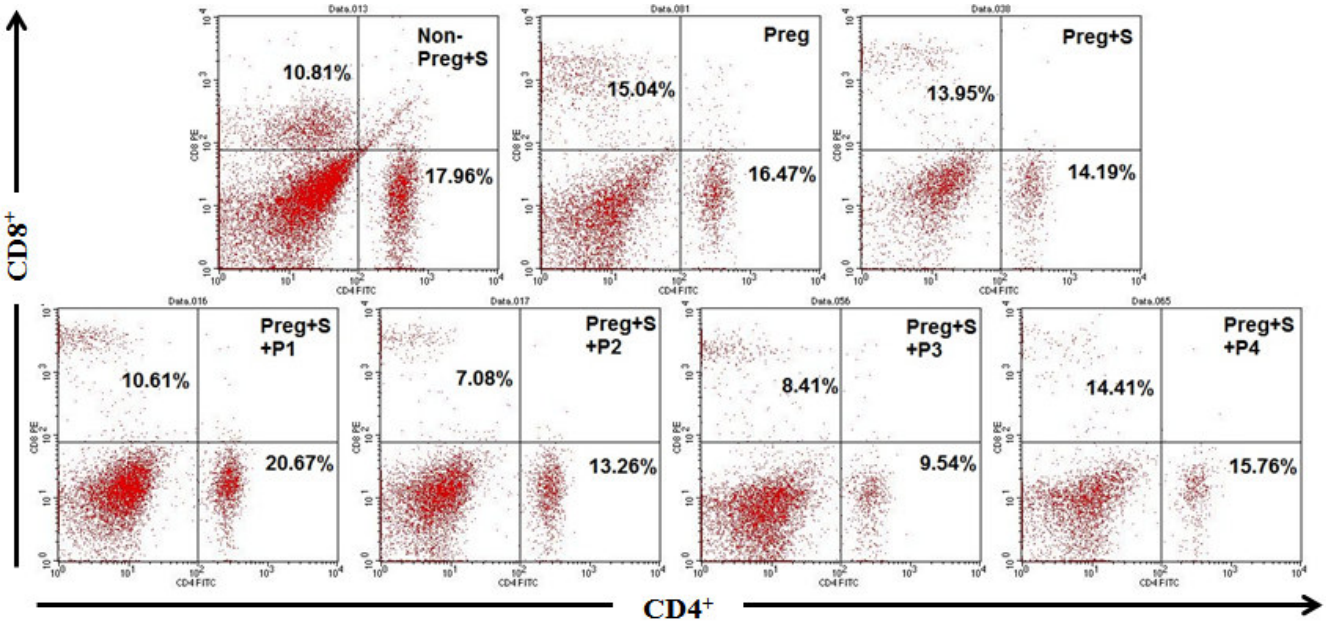

Figure 1

The Administration of E. scaber and P. obtusa Formulation for 14 days in pregnant mice infected by S. typhi modulate the expression level of $CD4^+$ and $CD8^+$ T cells

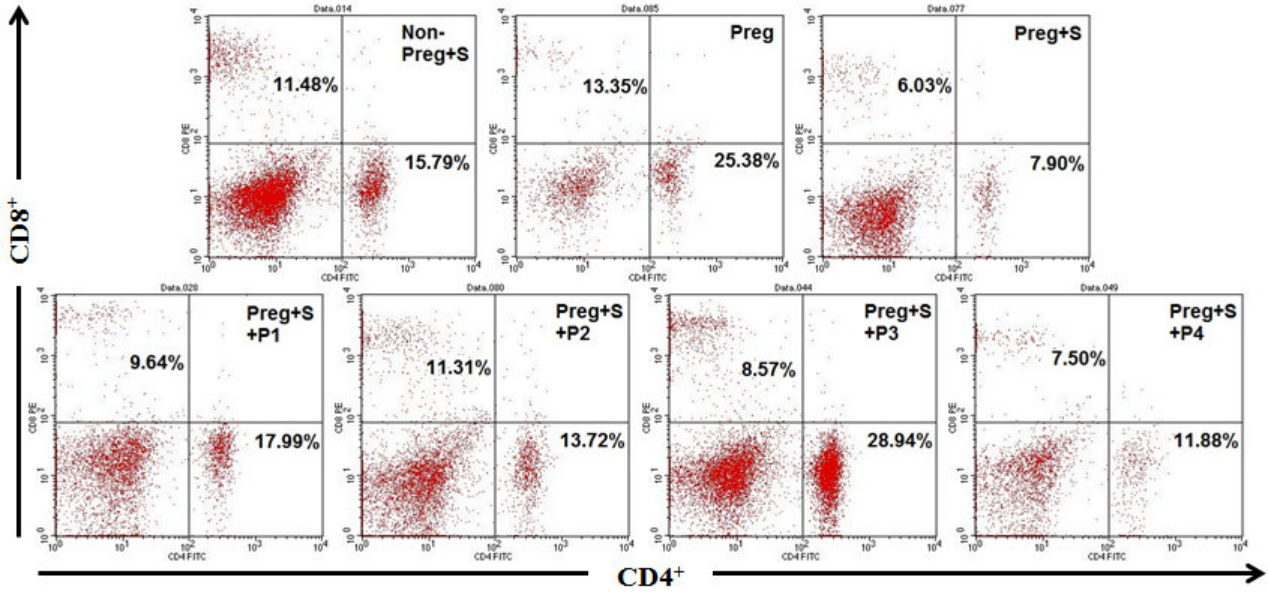

Figure 2

The Administration of E. scaber and P. obtusa Formulation for 18 days in pregnant mice infected by S. typhi modulate the expression level of $CD4^+$and $CD8^+$ T cells

The activated lymphocytes will lose molecule $CD62L^8$.The infection of *S. typhi* also reduced the number of CD4+CD62L- T cells both in non-pregnant and pregnant mice compared to pregnant mice without infection. This study proved that combination of *E. scaber* and *P. obtusa* leaf extract with the formulation P4 can activate CD4 T cells after 14 days of administration in pregnant mice infected by *S. typhi* (Figure 3). The modulation activity of combined *E. scaber* and *P. obtusa* leaf extract was also shown in Figure 4. The infection of *S. typhi* not only reduced the number of CD4+CD62L- T cells but also CD8+CD62L- T cells. In this research, the combination of *E. scaber* and *P. obtusa* leaf extract with the formulation of P1 and P2 had an ability to activate CD8 T cells after 18 days of administration in pregnant mice infected with *S. typhi*.

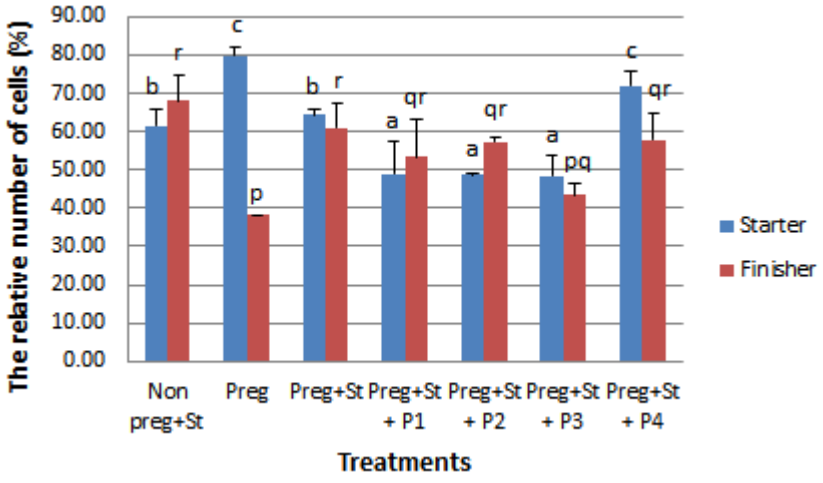

Figure 3

The Administration of E. scaber and P. obtusa Formulation in pregnant mice infected by S. typhi had efficacy to increase the relative number of $CD4^+CD62L^-$T cells

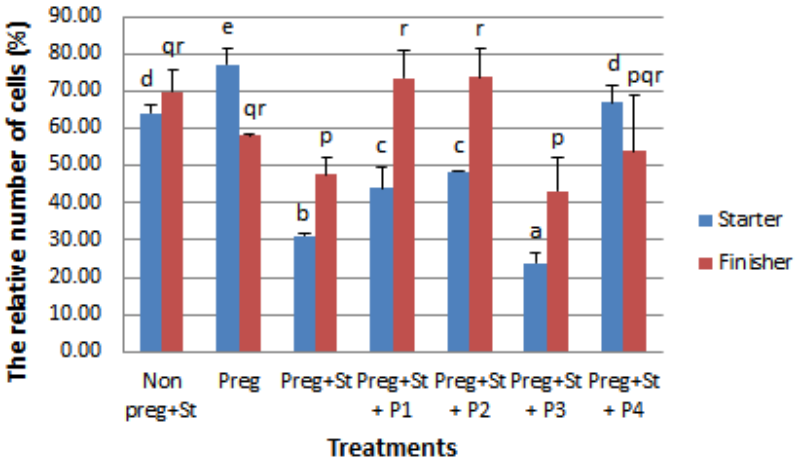

Figure 4
The Administration of E. scaber and P. obtusa Formulation in pregnant mice infected by S. typhi was able to increase the relative number of $CD8^+CD62L^-T$ cells

TER-119+ expression is obtained because it is the marker of mature erythrocyte from reticulocyte differentiation. Beside its role as mediator of respiration, erythrocyte also has a role in the immune system. It plays a role in the binding of antigen-antibody complex through complement receptor type 1 (CR1) on its surface[2]. As shown in Figure 5, the infection of *S. typhi* reduced the number of TER-119$^+$ cells both in non-pregnant and pregnant mice compared to pregnant mice without infection. This study demonstrated that the combination of *E. scaber* and *P. obtusa* leaf extract with the formulation of P1, P2, and P4 were able to activate TER-119$^+$ after 14 days of administration in pregnant mice infected with *S. typhi*. P3 formulation could activate TER-119+ after 18 days.

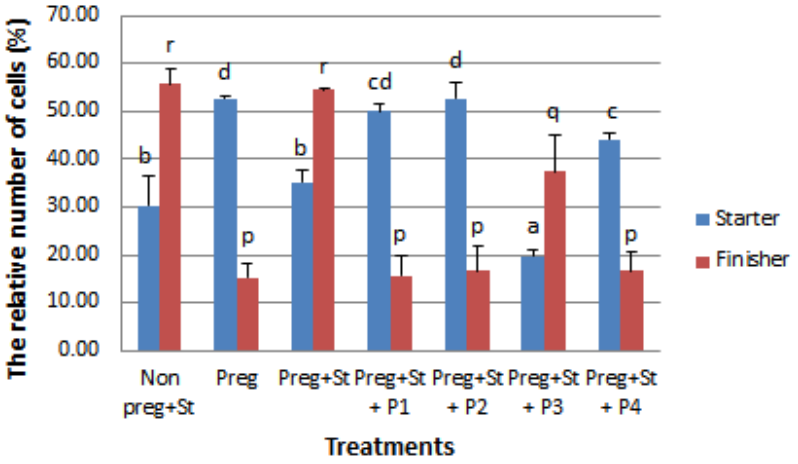

Figure 5
The Administration of E. scaber and P. obtusa Formulation in pregnant mice infected by S. typhi can increase the relative number of TER-119$^+$ cells

DISCUSSION

This study proves that *S. typhi* infection causes a decrease in the number of T lymphocytes both CD4$^+$ T cells and CD8+ T cells. The decrease mechanism of the number of T cells occurs due to *piroptotic* process. Ashida *et al*.[9] explained that the infection of pathogenic bacteria such as *S. typhi* will be recognized first by a group of proteins in the innate immune system of class Pattern Recognition Receptor (PRRs) which consists of PAMPs and DAMPs. The response of PAMPs and DAMPs can induce *inflammasome* formation consisting of NLRs, ASC, and caspase-1. *Inflammasome* trigger the secretion of pro-inflammatory cytokines such as cytokines IL-1β and IL-18, DNA fragmentation, and membrane rupture. Davis *et al*.[10] also explains that *piroptotic* is one of programmed cell death due to the presence of inflammation agents which mediate the *inflammasome* and activation of caspase-1. Ethanol extracts from all parts of *E. scaber* plant in phytochemical analysis show that this plant contains a number of secondary metabolites including *triterpenoidslupeol*, *sesquiterpene lactones* (*deoxyelephantopin*, *isodeoxyelephantopin*, 17.19-*dihydrodeoxyelephantopin*, *scabertopinisoscabertopin* and *elescaberin*), fatty acid esters (*ethylhexadecanoate*, *ethyl*-9, 12-*octadecadienoate*, *ethyl*-(Z)-9-*octadecenoate* and *ethyl octadecanoate*), *stigmasterol*, *stigmasterolglucoside*, *alkaloids*, *flavonoid*, *aurones*, *chalcones* and *phenolic* compounds in small quantities[11]. *P.obtusa* contains *polysciosida* A-

H, *flavonoids*, *saponin*, and *dexamethason* compound. The increasing number of $CD4^+$ and $CD8^+$ T cells after oral administration of formulation of *E. scaber* and *P. obtusa* leaf extract is assumed because *flavonoid*, *polysciosida* A-H, and *saponin* compounds from those plants play a role as an *immunostimulant*. *Immunostimulant* can increase the cell fission. Flavonoid has several benefits such as antibacterial agent, antiviral, anti-inflammatory, anti-angiogenic, analgesic, hepatoprotective, cytostatic, and anti-allergy[12]. According to Middleton *et al*.[13], flavonoid has a function to trigger Mitogen Activated Protein Kinase (MAPK) activity. MAPK is Protein Tyrosine Kinase (PTK) family which has a function to catalyze phosphorylation of cellular substrate and finally causes cell proliferation. Mitogen can stimulate the increase of IL-2 cytokine. IL-2 is an immunocompetent cell proliferation and differentiation factor. It will increase the concentration of cyclin D2 and E. Cyclin D2 and E are proteins which can activate the cyclin-dependent kinase (cdk). IL-2 has a function to inhibit p27 concentration which inhibits cdk complex. Based on that, IL-2 can induce cell from G1 to enter S phase in cell cycle so that cell will actively proliferate[14,15]. According to Weber *et al*. in Nworu *et al*.[16], MAPK also plays role in the activation of immune cell. It makes the number of active cell, in this case are $CD4^+CD62L^-$ and $CD8^+CD62L^-$ in pregnant mice infected by *S. typhi* which are given the formulation of *E. scaber* and *P. obtusa* orally higher compared to the other treatments. *Polysciosida* A-H is a compound from the *saponinoleanic* acid group. *Saponin* is a compound which plays role in flow process of nutrient in animals and human. It also plays role in membrane permeability, as *immunostimulant*, and *hypocholesterolaemic*[17]. It has a function to increase IFN-γ that produced by activate $CD4^+$ T cells ($CD4^+CD62L^-$). Lee *et al*.[18] and Shi *et al*.[19] mentioned that IFN-γ can stimulate up-regulation of MHC-II so that T cell will be differentiated into $CD4^+$ T cells. If the number of $CD4^+$ T cells is high then the number of CD8+ T cells is also high because IL-2 produced by $CD4^+$ T cells is not only used to up-regulate itself but also to up-regulate $CD8^+$ T cells. Furthermore, IFN-γ has a function to activate macrophage, increase phagocytosis capacity and cytolysis activity of natural killer (NK) cells[14]. Beside causing a decrease in the number of $CD4^+$ and $CD8^+$ T cells cells, the infection of *S. typhi* also caused a decrease in the number or mature erythrocyte ($TER\text{-}119^+$). It might occur because erythropoietin (EPO) hormone which was important in erythropoiesis process was decreased by infection. This statement is in line with Pui *et al*.[20] who explained that the *Salmonella* sp. bacterium produces a lipopolysaccharide (LPS) protein antigen, which plays a role in the invasion of the host cell to invade epithelial cells of the reticuloendothelial system (RES), in this case is the kidney. Hoffbrand[21] explained that 90% of EPO hormone is produced by the kidney *peritubular* interstitial cells and 10% is produced by the liver and other organs. Nairz *et al*.[22] proved that LPS can reduce EPO mRNA expression in the kidney. Decreased expression of EPO will have an impact on the decrease in the secretion of the EPO hormones so that erythropoiesis process will be disturbed and the number of mature erythrocytes ($TER\text{-}119^+$) in the bone marrow will decrease. An increasing number of $TER\text{-}119^+$ after oral administration of *E. scaber* and *P. obtusa* formulation is caused by the content of Fe or iron in *E. scaber*. That plant has been explored for the presence of trace elements such as Si, Ca, Cl, Mg, S, K, P, Al, Fe, Ti, and Sr[23]. *E. scaber* contains 45,4% of Fe. According to Linder[24], Fe or iron is needed in erythropoiesis process. In additional condition, Fe also can stimulate the synthesis of EPO hormone so Fe is also known as erythropoietic agent. Hoffbrand[21] explained that EPO can stimulate erythropoiesis by increased the number of progenitor of erythrocyte to proliferation and differentiation. This statement already answered why the administration of formulation P1 and P2 gave higher number of $TER\text{-}119^+$ compared to the formulation of P3 and P4 because P1 and P2 contained more *E. scaber* extract than *P. obtusa*. Besides, ethanol extracts of *E. sacber* have antimicrobial activity, so this plant can also be used to kill *S. typhi* directly. Ethanol extract of that leaves can stimulate wound healing activity which are characterized by decreasing chronic inflammatory cells, reduced swelling and increased *collagenation*[25]. Its efficacy in reduce inflammation can prevent cell from *piroptotic* led by *S. typhi* infection. Based on all of those mechanisms, the formulation of combined *E. scaber* and *P. obtusa* can work in synergy to cure *S. typhi* infection in pregnant mice by the role of immune system activation, direct killing on *S. typhi*, and the prevention in immunocompetent cell's death. However, those plants can also make the decrease of immune cell under some condition. According to Schroeter *et al*.[26], the action of flavonoid is very complex, sometimes synergism and sometimes antagonism depends on the specific component used, cell type, concentration, and experimental design. This study demonstrates that concentration of flavonoid and type of cell influences its effect to many kinds of cell.

CONCLUSION

This study demonstrates that the combination of *E. scaber* and *P. obtusa* plays a role in modulate immunocompetent cell especially in subset $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+CD62L^-$ T cells, $CD8^+CD62L^-$ T cells, and $TER\text{-}119^+$ cells during the infection of *S. typhi* in pregnancy condition. *E. scaber* and *P. obtusa* can help pregnant women maintain their immunity during infection. Those plants may play as an immune-modulator which can modulate immune competent cell's growth.

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