Anti-inflammatory Activity of *Elephantopus scaber* and *Sauropus androgynus* Combination in Pregnant Mice Infected with *Escherichia coli*

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Abstract

The current study aimed to investigate the effectiveness of *Elephantopus scaber* and *Sauropus androgynus* as anti-inflammatory agents in pregnant mice infected with *Escherichia coli*. This study used seven treatments group (*n*=3): K- (healthy pregnant mice), K+ (pregnant mice infected with *E. coli*), P1 (pregnant mice infected with *E. coli* + *E. scaber* 100%), P2 (pregnant mice infected with *E. coli* + *E. scaber* 75% and *S. androgynus* 25%), P3 (pregnant mice infected with *E. coli* + *E. scaber* 50% and *S. androgynus* 50%), P4 (pregnant mice infected with *E. coli* + *E. scaber* 25% and *S. androgynus* 75%), P5 (pregnant mice infected with *E. coli* + *S. androgynus* 100%). Flow cytometry analysis was used to analyze cell populations expressing $CD4^+TNF\alpha^+$, $CD4^+IFN\gamma^+$ and regulatory T cells ($CD4^+CD25^+CD62L^+$). All treatment groups significantly ($p<0.05$) decreased TNFα and IFNγ levels, while the P2 group was more effective in increasing regulatory T cells at the 1st and 2nd trimesters of the pregnancy. This study showed that *E. scaber* and *S. androgynus* combination alleviated inflammation by reducing inflammatory cytokines (TNFα and IFNγ) and increasing T-regulatory cells. Therefore, *E. scaber* and *S. androgynus* combination could suppress the inflammation during pregnancy and infection.

Keywords: *Elephantopus scaber,* immune system, infection, inflammation, *Sauropus androgynous.*

INTRODUCTION

The immune system protects the body from antigens like bacteria and viruses. Immunological pregnancy detection is essential for maintaining pregnancy and inadequate detection of fetal antigens leads to abortion [1]. These changes make pregnant women more susceptible to infectious agents. Pregnant women are considered a special group because of their specific susceptibility to several infections due to the unique immunological condition caused by pregnancy. Thus, pregnancy presents some challenges when deciding how to deal with, prevent, and treat infectious diseases [2].

The maternal immune system has many changes throughout the pregnancy period. These conditions protect the mother and fetus from pathogens while avoiding a detrimental immune response to the allogeneic fetus. Although there is little evidence that the maternal immune system is totally suppressed during pregnancy, the increased risk of certain infections indicates significant qualitative immunological changes [3]. Pregnancies are complex and unique circumstances. Therefore consideration should be given to understanding how specific endocrine, physiological and immunological factors increase the risk of infection. Specifically, urinary tract infections during pregnancy may occur more frequently, or pneumonia may become more severe, mainly due to decreased functional residual capacity of the lungs due to changes in the circulatory system and increased abdominal pressure) [4,5].

Several diseases may cause by bacterial infection [6]. Bacteria is one of the agents that can infect humans [6]. *Escherichia coli* is a pathogenic bacterium that can infect humans and cause various diseases [7]. During pregnancy, urinary tract infections are a widespread occurrence. The most common pathogen isolated from the urinary tract is *E. coli*. *Escherichia coli* has high resistance to Ampicillin, so it should not be used for *E. coli* infection therapy. Furthermore, some antibiotics harm the mother and fetus, like Pyelonephritis. Pyelonephritis can cause morbidity and be life-threatening to both the mother and fetus [8]. Placental infections by *E. coli* can produce inflammatory cytokines such as Tumor Necrosis Factor (TNF)-α, Interferon (IFN)-γ, Interleukin (IL)-12, and high levels of IL-6 activate the maternal immune system, leading to placenstal damage and miscarriage or premature birth [9]. Therefore alternative therapy is needed for *E. coli* infection in pregnant women.

Elephantopus scaber is a small herb native to the Neotropical Realms, Europe, Asia, Africa, and Australia. Sesquiterpene lactone compounds from *E. scaber* such as deoxyelephantopin,

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isodeoxyelephantopin, scabertopin, and isocabertin have emerged as potent anticancer compounds. Other biological activities, including antibacterial, hepatoprotective, antioxidant, anti-diabetic, and anti-inflammatory, were also reported [10]. Al Fahad *et al.* in 2012 showed that petroleum ether, chloroform, and methanol extracts from above-ground parts of *E. scaber* tested for antibacterial activity against *Staphylococcus aureus, Salmonella paratyphi A, Klebsiella pneumoniae, Pseudomonas aeruginosa, Shigella sonnei, Escherichia coli,* and *Salmonella typhimurium* have a significant activity to inhibit the growth of bacteria. However, methanol extract proved most effective against the organisms tested [11].

Sauropus androgynus, also known as *Katuk* in Indonesia, have some pharmacology activities [12]. Anju *et al.* recently showed that *S. androgynus* leaves are used as antitussives, tonics, and sedatives to the lungs and relieve internal heat [13]. The dark green leaves of *S. androgynus* contain valuable hematopoietic elements, cell rejuvenation, and the circulatory promoter chlorophyll. *S. androgynus* leaves were previously reported to have significant alkaloids in fresh leaves [14]. Selvi and Bhaskar confirmed that the leaves of *S. androgynus* may be beneficial in treating inflammation, pain, and fever [15]. These activities may be partly due to phytochemicals such as flavonoids, alkaloids, steroids, and terpenes.

Herbal medicine has been chosen as an alternative treatment for decreasing antibiotic utilization during pregnancy. The combination of *S. androgynus* and *E. scaber* is presumed to stimulate the immune system synergistically through anti-inflammatory effects. This study investigates the effectiveness of *E. scaber* and *S. androgynus* formulations as anti-inflammatory agents in pregnant mice with *E. coli* infection.

MATERIAL AND METHOD

Herbs Material

The powder leaves of *E. scaber* and *S. androgynus* were purchased from Balai Materia Medica Batu in Malang, Indonesia. Taxonomists have identified the leaves at Balai Materia Medica Batu in Malang. The leaf powder was put into a closed bottle and added 70% ethanol. Furthermore, the bottles were stored in a dark place overnight. The mixture was filtered and replaced with new ethanol and soaked again until the original color of ethanol was seen, indicating that the compound had been completely extracted. The extraction results were evaporated at a temperature of 50°C in a water bath using a vacuum pump evaporator (Brushless DC Motor). Each evaporation result in paste form was weighed according to the treatment dose and dissolved in aquadest for each day. The starting dose of *E. scaber* and *S. androgynus* was 200 and 150 mg.kg^{-1} body weight, respectively [16,17].

Animal preparation

This study used twenty-one female mice (*Mus musculus*) strain BALB/c as an experimental animal. The mice used are 4-5 weeks old with active health conditions, do not lose hair, and do not have deformed limbs. Mice were acclimatized for seven days since they arrived in Animal Laboratory, Biology Department, Brawijaya University Malang, Indonesia, from Gajahmada University, Yogyakarta, Indonesia. After acclimation, mice were mated with male mice, and a vaginal plug in female mice indicated day 1 of mice pregnancy.

Escherichia coli injection

Escherichia coli (EPEC) isolates were obtained from the Microbiology Laboratory, Faculty of Medicine, Brawijaya University, Malang, Indonesia. *E. coli* were injected intraperitoneally at five days of mice's gestation. The number of *E. coli* was 10^7 CFU.mL^{-1} in 0.5 mL per mouse. *E. coli* were detected using the Gram Staining and Catalase Test. *E. coli* infection was confirmed by collecting mice's tail vein blood after 24 h of infection.

Treatment of *E. scaber* and *S. androgynus* extract

The pregnant mice were randomly divided into seven experimental groups (n=3). All treatment groups are presented in Table 1. The treatment group consisted of control and extract treatment. The combination of *E. scaber* and *S. androgynus* leaf extract was administered orally from day 1 until day 16 of pregnancy.

Table 1. Experimental groups (*n*=3)

Group	*E. coli**	ES extract	SA extract
K-	-	-	-
K+	+	-	-
P1	+	200	-
P2	+	150	37.5
P3	+	100	75
P4	+	50	112.5
P5	+	-	150

Note: *Infected of 10^7 CFU.mL^{-1} *E. coli*, ES= *Elephantopus scaber* (mg.kg^{-1} BW); SA= *Sauropus androgynus* (mg.kg^{-1} BW).

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Cell isolation

Mice were dissected in 1st, 2nd, and 3rd trimesters of pregnancy. Lymphocyte cells were isolated from the spleen. Spleen squeezed with the tip of the syringe, crushed clockwise, and suspended with PBS. Furthermore, the obtained cells were filtered. The crushed spleen was centrifuged at a speed of 3200 rpm at a temperature of 20°C for 5 min. The supernatant was discarded, and the pellet was resuspended with 1 ml of PBS. A total of 100 µL homogenate was transferred to a new microcentrifuge tube, and 500 µL of PBS was added. Then it was centrifuged at 3200 rpm at 20°C for 5 min. The obtained pellet was stained with the specific antibody.

Flow cytometry analysis

Flow cytometry was used to analyze the cell populations expressing $CD4^+TNF\alpha^+$, $CD8^+IFN\gamma^+$, and $CD4^+CD25^+CD62L^+$. Firstly, the obtained pellet was stained with extracellular antibodies (FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD25, and PE-conjugated rat anti-mouse CD62L) provided by Biolegend, San Diego, CA, and then incubated for 20 min at 4°C. Subsequently, the cells were added with 50 µl fixative solution (cytofix/cytoperm) and incubated for 20 min at 4°C. The residual of the fixative solution was removed by washing solution (washperm) and then centrifuged at 2500 rpm at 10°C for 5 min. The supernatant was discarded and pellets were stained with intracellular antibodies (PE-conjugated rat anti-mouse TNFα, and PE/Cy5-conjugated rat anti-mouse IFNγ) provided by Biolegend, San Diego, CA, and then incubated for 20 min at 4°C. The sample was then transferred to a flow cytometry cuvette and pipetted. Flow cytometry analysis was carried out using a flow cytometer BD FACS Calibur™ and a computer that was installed with the BD Quest Pro™ software.

Data analysis

The data were analyzed using BD CellQuest PRO™ software and then tabulated. One-way ANOVA was used for statistical analysis with a $p<0.05$. Statistical analysis was performed using SPSS version 16.0 for Windows.

RESULT AND DISCUSSION

TNFα is a proinflammatory cytokine secreted by $CD4^+$ T cells, $CD8^+$ T cells, B cells, and macrophages. This study determined the relative number of TNFα produced by CD4+ T cells. The relative number of $CD4^+TNF\alpha^+$ in the infected pregnant mice (K^+) significantly increased compared to the healthy pregnant mice (K-) (Figure 1).

The results also demonstrated that P1-P4 groups significantly ($p<0.05$) reduced the relative number of $TNF\alpha^+$ compared to the K^+ group. The lowest decrease was found in the P1 and P2 groups. However, the P1 and P2 were not significantly different in reducing TNFα levels produced by CD4 T cells. Combining *E. scaber* and *S. androgynus* is expected to relieve inflammatory conditions by decreasing TNFα levels. *Sauropus androgynus* has been known to contain high flavonoid content. Gresso [18] showed that flavonoids could reduce LPS-induced TNFα levels through phosphorylation of p38 MAP kinase.

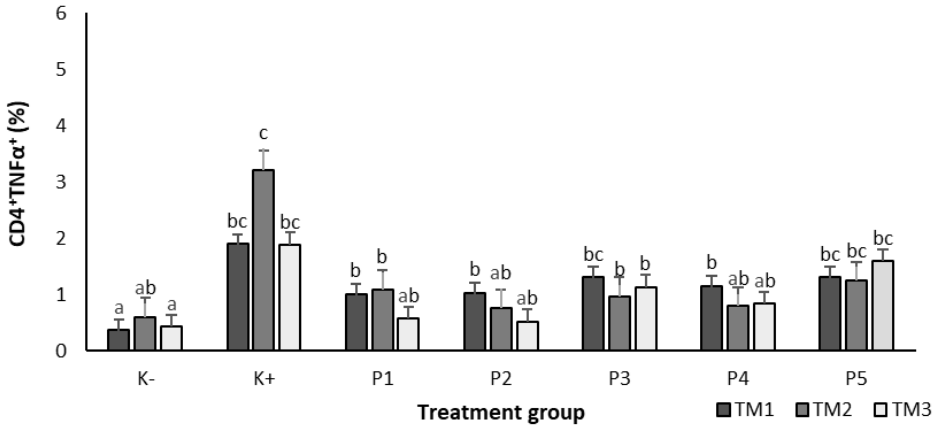

Figure 1. The relative number of TNF-α produced by $CD4^+$ T cells in all treatment groups. Mice were dissected in 1st, 2nd, and 3rd trimesters of pregnancy. Lymphocytes were isolated from the spleen and then analyzed with flow cytometry. Data were presented as mean ± SD from 3 mice in each treatment group with $p < 0.05$.

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The increase in the TNFα was also accompanied by the rise in other proinflammatory cytokines, such as IFNγ. IFNγ is a proinflammatory cytokine that plays a vital role in inflammation. Therefore, reducing the relative number of IFNγ is expected to reduce inflammation. Figure 2 showed that all treatment groups (P1-P5) significantly (p<0.05) suppressed the production of IFNγ in CD4 T cells compared to the K+ group. Vitamins A and E in *E. scaber* contribute to inhibiting proinflammatory cytokines by Th2 cells [16].

The results showed that peritoneal injection of *E. coli* caused a significant decrease in the relative number of regulatory T cells in the K^+ group compared to the K- control group (Fig. 3). The combination of *E. scaber* and *S. androgynus* significantly increased the relative number of regulator T cells (p<0.05) in all treatment groups compared to the K+ group at all trimesters. The highest increase in the relative number of regulatory T cells was found in the P2 group (*E. scaber* 75% and *S. androgynus* 25%. *Sauropus androgynus* contains a lot of tannins, saponins, flavonoids, and alkaloids, which can trigger MAPK activity to stimulate Treg production [15].

The function of the immune system is to protect the host from pathogens. This function depends on the ability of the innate immune system to regulate cell migration to monitor, recognize and respond to invading microorganisms. Mor *et al.* showed that 70% of decidua leukocytes are NK cells, 20-25% are macrophages, and 1.7% are dendritic cells [2]. Although B cells are absent in the adaptive immune system, T lymphocytes comprise about 3-10% of decidual immune cells [19].

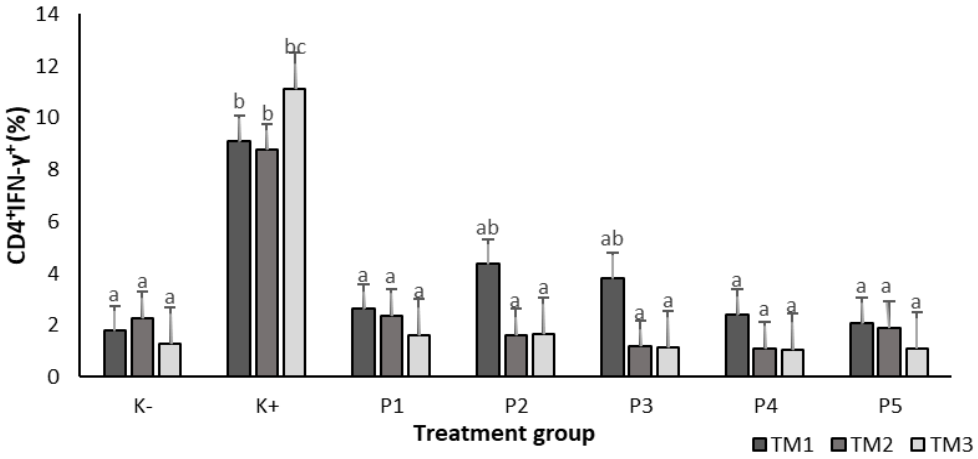

Figure 2. The relative number of IFN-γ^+ produced by $CD4^+$ cells in all treatment groups. Mice were dissected in 1st, 2nd, and 3rd trimesters of pregnancy. Lymphocytes were isolated from the spleen and then analyzed with flow cytometry. The data presented as mean ± SD from 3 mice in each treatment group with $p < 0.05$.

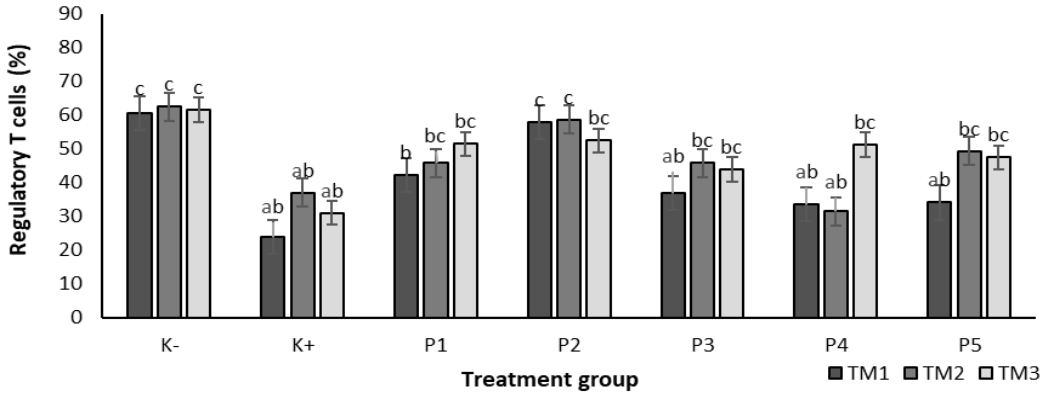

Figure 3. The relative number of regulatory T cells ($CD4^+CD25^+CD62L^+$) in all treatment groups. Mice were dissected in 1st, 2nd, and 3rd trimesters of pregnancy. Lymphocytes were isolated from the spleen and then analyzed by flow cytometry. The mean ± SD value from 3 mice in each treatment group ($p < 0.05$).

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In early pregnancy, NK cells, dendritic, and macrophages infiltrate the decidua and accumulate around the invading trophoblast cells [20]. Deletions of macrophages, NK cells, or dendritic cells (DCs) have adverse effects [21]. Hanna *et al.* studies have shown that in the absence of NK cells, trophoblast cells cannot reach the endometrial vascular distribution, leading to abortion. These studies suggest that NK cells are important for uterine endometrial infiltration [22]. Similarly, DC depletion prevented blastocyst implantation and decidua formation. DC is required for decidua formation and may affect the angiogenic response by inhibiting vascular maturation [23].

This study proves that the combination of *E. scaber* and *S. androgynus* has anti-inflammatory activity. *E. scaber* contains many saponin and flavonoid compounds. Flavonoids and saponins have an essential role in suppressing inflammation. *Sauropus androgynus* extract had activity in reducing inflammation. Activation of cyclooxygenase can increase prostaglandins, especially PGE2, and this production can inhibit inflammation, pain, and fever [24]. Ginwala *et al.* showed that flavonoids have an anti-inflammatory role through several different mechanisms as inhibitors of regulatory enzymes and transcription factors that have essential functions in mediating inflammation [25]. Flavonoids also have antioxidant potential that can reduce free radicals and their formation. So, flavonoids have an important influence on immune mechanisms in the inflammatory process [25].

Flavonoids can inhibit protein kinase as an anti-inflammatory mechanism. Protein kinases are proteins involved in signal transduction during cell activation in inflammation. Certain flavonoids can target several kinases through several mechanisms [26]. The decreased T-reg cells in sick mice K^+ was associated with increased cytokines, including IL-6, TNF-α, and IFN-γ. Usually, the presence of TGF-β will induce naive T cells to become Treg cells, but the presence of IL-6 will change this function. IL-6 and TGF-β together cause the differentiation of naive T cells into Th17 cells and the formation of inactivated Treg cells so that the number of Treg cells decreases. It is related to Dienz and Rincon, who described IL-6 as a proinflammatory cytokine that could inhibit the differentiation of naive T cells into TGF- induced Tregs [27].

Another research also reported that a combination of *S. androgynus* and *E. scaber* possessed antibacterial activity during pregnant and infection. Christina *et al.* [28] stated that a ratio of 75:25 exhibits significant protection in the renal and hepatic of infected pregnant mice. An equal ratio of both extracts also decreased the inflammation mediated by granulocyte (Gr-1). The combination of *S. androgynus* and *E. scaber* also have the potential effect as a hormonal balancer in infected pregnant mice. Djati *et al.* [29] also reported that *S. androgynus* and *E. scaber,* at a ratio of 75:25, also altered the hormonal changes and erythropoiesis in infected pregnant mice. Therefore, *E. scaber* and *S. androgynus* combination could prevent damage from bacterial infection during pregnancy.

CONCLUSION

This study showed that *E. scaber* and *S. androgynus* combination alleviated inflammation by reducing inflammatory cytokines (TNFα and IFNγ) and increasing T-regulatory cells. Therefore, *E. scaber* and *S. androgynus* combination could suppress the inflammation during pregnancy and infection.

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