

The Effect of *Elephantopus scaber* Extract to TNF- α and TGF- β on Mice under Carcinogen Treatment

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Abstract

Carcinogen compounds are the compounds that cause DNA damage and trigger cancer. TNF- α and TGF- β are cytokines produced by immune cells and serve to maintain body homeostasis. *Elephantopus scaber* is a plant that prevents the cancer progression, and improve the body's immune system, so this study was conducted to determine the effect of *E. scaber* extract on TNF- α and TGF- β after the administration of carcinogen compounds. The study was conducted by administering the carcinogen compound DMBA with a dosage of 0.56 mg kg⁻¹ BW and estradiol with a dose of 0.0504 mg kg⁻¹ BW which was given alternately for 8 weeks to the *Mus musculus* test animals. The study was conducted in 3 groups: K- (the normal group of mice), K + (a carcinogen-induced group of mice), and P (a group of carcinogen-induced mice and *E. scaber* extract). The treatment was done in 1 week, 2 weeks, and 3 weeks. Immune cells were isolated from splenocytes and performed immunostaining for flow cytometry analysis. The computed relative amount is TNF- α produced by macrophages CD11b and TGF- β produced by Treg CD4CD25. The relative number of cells was analyzed by two-way ANOVA and advanced Tukey test with 95% confidence level ($\alpha = 0.05$). The results showed no significant differences in the number of cytokines TNF- α and TGF- β in both the carcinogen-induced mice group and the mice group was given the extract of *Elephantopus scaber* for 1 week, 2 weeks and 3 weeks.

Keywords: Carcinogen, *Elephantopus scaber*, TNF- α , TGF- β .

INTRODUCTION*

Carcinogens are various substrates that can cause cancer or carcinogenesis. Carcinogenic compounds do not always cause direct poisoning, but can be in a very long time [1]. When the carcinogen compound enter the body, it will be detoxified by the liver through a biotransformation process to transform the toxic compounds to become water-soluble so it can be easily removed from the body. However, in some cases, this process can change the non-toxic compounds to be more toxic [2].

One of the most carcinogenic compounds that widely used in cancer modeling research was DMBA (7,12-Dimethylbenz [a] anthracene), the immunosuppressor compound and potent cancer-promoting agent [3]. The DMBA bioactivation process on microsomal P-450 by NADPH produced 7-Hydroxymethyl-12-methylbenz [a] anthracene (7-HMBA) metabolite which then cytosolic sulfotransferase by PAPS into more mutagenic compound 7-HMBA sulfate [4]. Mutagenic compounds which have the potential to damage DNA occurs to cause errors during the process of replication, resulting in the occurrence

of mutations and alteration epigenetic [5]. Such epigenetic mutations and alterations may be replicated as cells proliferate, thereby altering gene function or expression of the gene that would potentially be a cancer-triggering agent.

Previous research has shown that increased DNA damage on cultured cells was responded by activating oncogenic factors [6]. Stress at the time of cell replication coincides with the inactivation of mutated gene caused by DNA damage, lead to downregulation or loss of DNA damage response mechanisms, resulting in cell loss of ability to perform DNA repair, senescence, and cell death programs (apoptosis), and trigger the occurrence of cancer [7]. The body has a natural response to control the repairing process caused by DNA damage. One of the body's natural factors that act as the body defending system is immune cells.

The immune system has the ability to detect the presence of cell abnormalities in the body, and provides a natural response to apoptosis programs so that the balance of the body defending system is maintained [8]. Through pro-inflammatory and anti-inflammatory responses, the immune system works to induce body homeostasis. One of the cytokines that play a role in this process is TNF- α and TGF- β . Tumor necrosis factor α (TNF- α) is a signal protein that plays an important role in inflammatory regulation, and produced most by macrophages

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[9]. TNF- α can induce cell death pathways by activating NF- κ B, activating MAPK pathway or by activation of caspase-8 [10,11,12].

In addition to TNF- α , Transforming Growth Factor β (TGF- β) is also a cytokine that plays an important role in the regulation of immune system. TGF- β is anti-inflammatory cytokine that produced by all the immune cells, which serves to induce gene transcription for differentiation, chemotaxis, proliferation, and activation of immune cells [13,14]. Both TNF- α and TGF- β are important signal proteins to maintain body immune system through inflammatory and anti-inflammatory responses.

One of the plants that have anti-inflammatory activity is *Elephantopus scaber* [15]. *E. scaber* has long been used in traditional medicine in Brazil [16]. *E. scaber* contains deoxyelephantopin active compounds that are able to inhibit the growth of HCT116 collecting cancer by inducing apoptosis and cell cycle retention [17]. Deoxyelephantopin is also capable of inhibiting the growth of breast tumors and the proliferation of lung cancer metastases [18]. Deoxyelephantopin was able to fight cancer by inducing the activation of PPAR- γ (Peroxisome proliferator-activated receptor gamma), inhibiting NF- κ B, has antiproliferation effects, and inducing apoptosis in tumors [19]. Due to some of the things mentioned earlier, in this study researchers wanted to know the effect of *Elephantopus scaber* plant extract on relative number of TNF- α and TGF- β in mice under carcinogen compounds treatment.

MATERIAL AND METHOD

Animal Trial

The experiment animals used in this study were BALB/c female mice with an average weight of 15-20 grams aged 5 weeks and obtained from Malang Murine Farm, Singosari. The procedure for the use and maintenance of experiment animals in this study is in accordance with the procedure and has been approved by the Research Ethics Committee of Brawijaya University with no. 648-KEP-UB.

Elephantopus scaber Extract

E. scaber leaf simplicia was obtained from Materia Medica Batu, extracted with 96% ethanol. The extract with a dose of 50 mg kg⁻¹ BW then dissolved in warm distilled water 0.5 mL volume.

Carcinogen Treatment

Mice were given DMBA carcinogen with a dose of 0.56 mg kg⁻¹ BW obtained from Sigma

Aldrich company, and injected subcutaneously in the mammary gland area with a 4 mm diameter syringe needle. Injection was done 4 times in 8 weeks. To accelerate the occurrence of cancer, sex hormone estradiol was also added. Estradiol is an estrogen, which functions for the growth and differentiation of reproductive organs [20], so in this study estradiol was used as an induction to accelerated the proliferation of mammary glands to formed cancer cells. Estradiol was obtained from the company Tokyo Chemical Industry (TCM), dissolved in corn oil with a dose of 0.0504 mg kg⁻¹ BW. Injection of estradiol was performed subcutaneously in the mammary gland area with a 4 mm diameter syringe needle. Injection of estradiol was performed 4 times in 8 weeks given alternately with DMBA.

Elephantopus scaber Extract Treatment

E. scaber leaf extract was given after the induction of carcinogen compound DMBA and Estradiol has finished. Based on previous research [21], the optimal dose of *E. scaber* extract to maintain immune system was 50 mg kg⁻¹ BW, the extract was administered orally with cannula (sonde) with a total volume of 500 μ L. The extract was administered daily for 1 week, 2 weeks, and 3 weeks.

Flow cytometry Analysis

Mice was sacrificed by neck dislocation, then the spleen organ was obtained. The spleen organ crushed with cold PBS to collected spleenocytes. The collecting cells then was washed until there is no residue remained. The resulting cells were extracellularly stained by adding 50 μ L of monoclonal antibodies obtained from Biolegend, i.e. Fluorescein Isothiocyanate (FITC) anti-mouse CD11b, CD4, CD25 with a concentration of 0.5 mg mL⁻¹ respectively and then incubated in cold dark spaces.

Before Intracellular staining was performed, the Fixation buffer (Paraformaldehyde 4%) of Biolegend was added to the suspension and incubated in dark cold spaces. After that added the Permeabilization Wash Buffer (wash/ferm) from Biolegend to wash the remaining of the fixation buffer. Then the cells were stained with intracellular antibodies from Biolegend, ie PE anti-mouse TNF- α and TGF- β Antibody with each concentration of 0.2 mg mL⁻¹ and incubated in dark cold spaces. The stained sample were then analyzed by flowcytometry BD FCS Calibur. Furthermore, acquire is selected and will count the total cell count as well as the number of cells

detected by the antibody label. The results obtained are then processed with BD cellquest ProTM software.

Data Analysis

Data presented and analyses statistically by two way Anova with significance $p < 0$.

RESULT AND DISCUSSION

The induction of DMBA and Estradiol carcinogen compounds for 8 weeks induced the formation of breast cancer

7,12-Dimethylbenz[a]anthracene (DMBA) was a carcinogen compound that has an aromatic hydrocarbon chain and has been widely used in research as an inducer in breast cancer modeling [22,23,24]. Estradiol is a steroid the sex hormone that useful in female reproductive cycle in regulation of estrous and menstrual cycles. Estradiol has functions as transcription factor and gene expression, the bonding of estradiol with both uncontrolled receptors can trigger the development and progression of cancer cells such as breast cancer, ovarian cancer, and endometrial cancer [25-28]. In addition, estradiol along with progesterone regulates proliferation of breast epithelial cells, thus inducing the occurrence of breast cancer [29]. In this study, breast cancer was formed after induction of DMBA and Estradiol for 2 months.

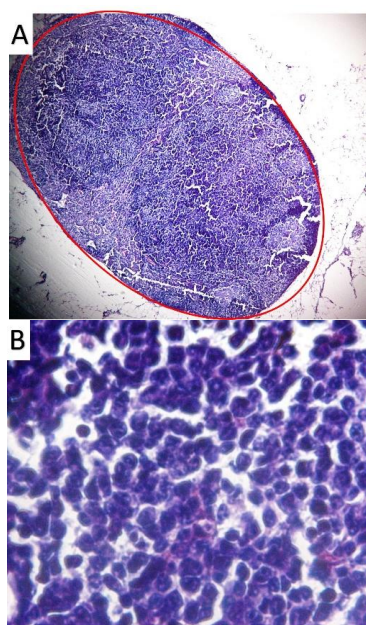


Figure 1. Histologic observation of mice breast tissue induced by carcinogen for 8 weeks at 40 times (A), and 1000 times (B) magnification with H & E staining. Higher cell density appears in red circled areas, indicating high cell proliferation or neoplasm. The resulting neoplasm was solid lobular carcinoma.

Neoplasm is an abnormal cell growth and often referred as a tumor, which formed a mass [30,31]. Provision of carcinogen compounds for 8 weeks in this study induced the formation of neoplasms in mice breast tissue. Based on histologic observations (Fig. 1), some breast tissue mice have transformed into a solid invasive lobular carcinoma. This carcinoma was derived from the lobular ducts, but the cells were mixed and ducts were no longer visible, made it hard to distinguish its cell types.

Invasive lobular carcinomas have characteristics such as round-to-polygonal cells with fewer cytoplasmic ratios when compared to nuclei (enlarged nucleus), small cells with undifferentiated nuclei and cytoplasm, mitotic and hyperchromatin images rarely seen, cell cohesion low, normal epithelial cell layer has been ruined by the proliferation of neoplastic cells above the pagetoid spread. The use of E-cadherin in immunohistochemical staining will make it easier to distinguish lobular carcinoma with the carcinoma ducts [32].

E. scaber extract treatment does not affect the relatives number of TNF- α and TGF- β

TNF- α is an endogenous pyogenic that plays an important role in immune cells regulation, to induces fever, cell death (apoptosis), cachexia, inflammation, and to inhibit tumorigenesis [33]. In this study, TNF- α cytokine profiles of CD11b macrophages were observed to investigate the effect of *E. scaber* extract on inflammation response induced by carcinogenic compounds.

TGF- β is an anti-inflammatory cytokine which under normal conditions serves to stop the cell cycle in G1 phase, and induces apoptosis. TGF- β can suppress the epithelial cell proliferation and cancer growth in early stage, and inactivated mutations in some tumors through TGF- β signaling pathway [34,35,36]. In the case of severe cancer, TGF- β undergoes mutation and loss of control, thus becoming an immune-suppressor agent, and inducing angiogenesis that causes the cancer to become more invasive [37]. In this study, the relative amount of TGF- β was observed to investigate the effect of carcinogenic compounds induction as well as the effect of *E. scaber* extract on TGF- β relatives number fluctuations.

The results showed that there were an increasing number of TNF- α cytokine by macrophage CD11b in the K + mice group of 6.95% when compared with the K- group of K- by 4.58% in the first week (Fig. 2A). However, the

differences occurred in the second and third weeks in which TNF- α in the K + group mice decreased by 6.37% and 6.48% respectively, when compared to the K-groups of 10.75% and 16.16%, respectively. The administration of *E. scaber* extract in group P for 1 week and 2 weeks increased TNF- α profile by 14.28% and 15.92% (Fig. 2A).

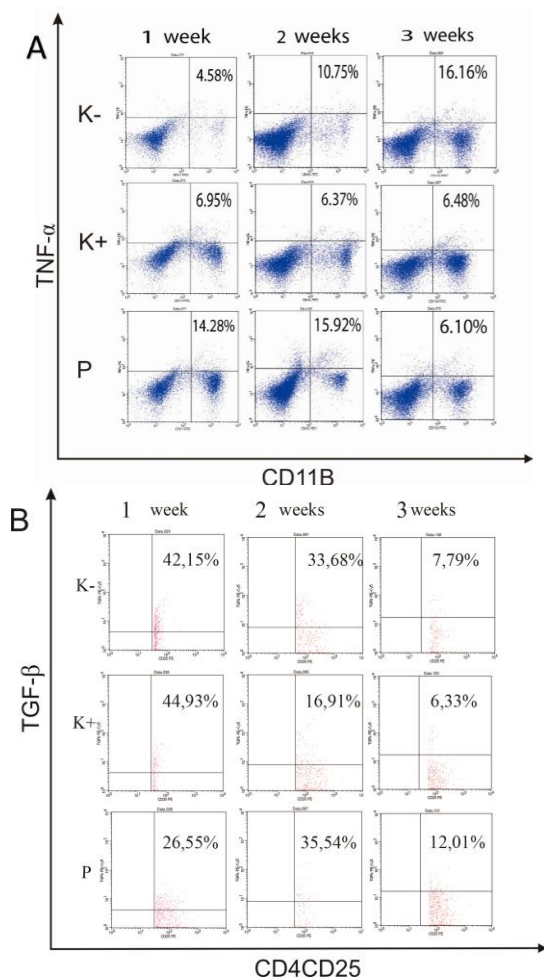


Figure 2. The flow cytometry results of the relative number of TNF- α (A) and TGF- β (B) isolated from macrophages and Treg, with K- (normal group), K + (cancer group with carcinogen induction), P (group of cancer-treated mice extract of *E. scaber*).

The result of the relative number (%) of TGF- β in flow cytometry was obtained by purifying CD4 and CD25 T cell, after which, the cell expressed CD4CD25 was purified again to obtained CD4CD25 T cells that expressed TGF- β . The results showed that in the K + mice group from 1 week to 3 weeks the relative number (%) of TGF- β ranged from 7.79% to 42.15%. While in group of K- week 1 to week 3 ranged between 6.33% - 44.93%, and in group of P week 1 to week 3

ranged from 12.01% -35.54%. The number of TGF- β continues to decrease weekly (Fig. 2B).

Based on statistical analysis using Tukey test, there were no significant difference in relative number (%) of TNF- α based on treatment group and time of extract with significance ($p < 0.05$) (Table 1). In the TGF- β cytokine, there was a relative decreased in the number of relative (%) at week 3 by $8,712 \pm 2,531$ when compared with the relative number of (%) TGF- β at week 1 and 2 each of which were $37,877 \pm 5,512$ and $28,708 \pm 2,639$ (Table 1). Based on the treatment group, there was no significant difference in the relative number (%) of TGF- β between the K-, K +, and P mice (Table 1).

Table 1. The relative number (%) of TNF- α and TGF- β after administration of *E. scaber* for 1 week, 2 weeks, and 3 weeks

	TNF- α	TGF- β			
		E. scaber treatment			
		1,2,3, weeks	1 week	2 weeks	3 weeks
K-					
K+	9.73 ^a		37,88 ^b	28,71 ^b	8,71 ^a
P					

Notes: The relative numbers (%) of TNF- α and TGF- β based on duration of 1 week, 2 weeks, and 3 weeks, with K- (normal group), K + (cancer group with carcinogen induction), P (group of cancer-treated mice extract of *E. scaber*), Signification ($p < 0.05$).

Tumour Necrosis Factor- α (TNF- α) is a pro-inflammatory cytokine produced by macrophages, was a protein involved in the inflammatory process, and cause hemorrhagic necrosis to induce tumor cell death. TNF- α activate the 3 different cell responses, namely cell defense and proliferation, pro-inflammatory gene transcription, and cell death. TNF- α has 2 receptors, TNFR1 and TNFR2. Binding of TNF- α to its receptor TNFR1 leads to activation of transcription factor NF- κ B, pro-inflammatory gene, and self defense. In other cases, TNF- α bound to its receptor TNFR2 may initiate the IKK phosphorylation that causes translocation of NF- κ B via the same signal path as TNFR1. What distinguishes TNFR1 signal pathway with TNFR2 is TNFR2 can activate endothelial/epithelial tyrosine kinase Etk involved in adhesion, migration, proliferation, and cell defense. In endothelial cells, TNFR-2 activates Etk via tyrosine phosphorylation of the intracellular VEGFR2 resulting in a single VEGF involved in the angiogenesis response by activating Akt [38].

In other hand, TNF- α was also a factor that caused cachexia, which is wasting syndrome that makes the sufferer lose weight, muscle atrophy, excessive fatigue, and the body becomes very weak [39]. Approximately 50% of cancer patients suffer from cachexia, this is because the condition of cancer can cause symptoms cachexia which is a side effect of chemotherapy that causes decreased quality of life, and is considered a cause of death in many cases of cancer patients [40,41].

In this study, there were no significant fluctuations in the relative numbers of TNF- α in both carcinogenic induced or in groups with *E. scaber* extract treatment (Table 1). Based on this results, the researcher assume that cancer induction by carcinogen compounds DMBA and Estradiol for 2 months has not resulted in excessive inflammatory reactions to cause cachexia syndrome. In rats model, DMBA can cause cachexia after 195 days of administration, while in 1 month administration of DMBA was not enough to promote cachexia syndrome [42,43]. TNF- α is cytokines that play major role to cause cachexia syndrom, and the increasing number of TNF- α can be considered as cachexia symptoms, because TNF- α responsible to degradation of myofibrillar and soleus muscle proteins [44]. The administration of *E. scaber* extract did not affect the inflammatory response, because it contains various phenolic compounds that have antioxidant and anti-inflammatory functions [45].

The immune system works in harmony interconnected with each other, where the function of immune cells will not work properly without other factors affecting it, and immune cells work to influenced each other [34,35,36]. Carcinogenic compounds induction by DMBA and estradiol not only affect the pro-inflammatory cytokines alone but also the anti-inflammatory cytokine expressed by CD4CD25 Treg cells.

Transforming Growth Factor- β (TGF- β), is a multifunctional cytokine which is a protein signal and is produced by many types of immune cells, including Treg [34]. TGF- β under normal conditions serves to stop the cell cycle in G1 phase, and induced apoptosis. TGF- β suppressed epithelial cell proliferation and early cancer growth, and may inactivated mutations in some tumors via TGF- β signal [46]. TGF- β in the early stage of cancer formation, serves to stop the cell cycle in G1 phase and induce cell apoptosis [46]. TGF- β otherwise secreted by Treg cells, was also

secreted by cancer cells itself to attract Treg cells [37].

In this study, the relative number (%) of TGF- β continued to decline weekly (Table 1), but the induction of carcinogenic and *E. scaber* extract did not affect the fluctuations of TGF- β relative number (%) produced by Treg CD4CD25 (Table 1). Because of this, researchers assume that carcinogenic compounds DMBA and Estradiol induction for 2 months was in early stage induction of cancer so it has not affected the relative number of TGF- β , because in more advanced cases, the TGF- β was usually increased because it also produced by the tumor cells themselves to attract Treg and as an immuno-suppressor to escape from the immune cells [47].

In the case of severe cancer, the TGF- β undergoes mutation and loss of control, thus becoming an immuno-suppressor agent, and inducing angiogenesis that causes the cancer to become more invasive [40,41]. TGF- β has a very important and crucial role in maintaining cellular homeostasis. In normal epithelial cells, TGF- β plays a role in antiproliferation response, promotes cell differentiation, and cell apoptosis induction. Thus TGF- β has a very important role as an anti-proliferative agent in early stage cancer [44].

Based on relative number of TNF- α and TGF- β fluctuation, the administration of DMBA and Estradiol for 1 week, 2 weeks, and 3 weeks does not cause severe inflammation response, but the inflammation response does not affected only by TNF- α and TGF- β , various number of pro-inflammatory and anti-inflammatory cytokines involved in inflammation response. Further examination is necessary to find out the inflammatory response caused by carcinogenic compound DMBA and Estradiol, and the various dose of *E. scaber* extract administration is necessary to find out the most effective dose of *E. scaber* extract that have anti-inflammation effect against carcinogenic induction.

CONCLUSION

The induction of carcinogen compounds in the form of DMBA and Estradiol for 8 weeks can trigger the occurrence of breast cancer, but has not been able to change the fluctuations in the relative number of cytokines TNF- α and TGF- β produced by macrophages CD11b and Treg CD4CD25. Administration of *E. scaber* extract for 1 week, 2 weeks, and 3 weeks did not change the relative number of the TNF- α and TGF- β

cytokines produced by CD11b and Treg CD4CD25 macrophages.

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