

Research Article

## Immunomodulatory Activity of Methanol Leaf Extract of Neem (*Azadirachta indica* Juss) Against Suppressor and Proinflammatory Molecules

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**ABSTRACT**

Neem plant is rich in bioactive constituents, which make it massively discussed the treatment of various diseases. A study on the immunomodulatory activities of neem is given here. This current work aimed to investigate the effects of neem leaf extract on immunocompetent cells. In vivo experiment was carried out using mice (*Mus musculus*) induced with DMBA, comprising positive control, negative control, and treatments of neem leaf extracts (250, 500, and 1000 ppm). Data obtained from flow cytometric analysis were evaluated using BD Cellquest Pro™ software, then statistically analyzed in SPSS version 21. Parametric analysis in one-way ANOVA was performed at a significance level of 5%. The significant difference was compared in the Duncan test. The results showed that administration of neem leaf extracts significantly affected the expression of CD4<sup>+</sup>, CD8<sup>+</sup>, CD25<sup>+</sup>, CD62L, IL-10, and IL-17 cells. Neem leaf extract has immunomodulatory activities by increasing pressure molecules and decreasing pro-inflammatory molecules.

*Keywords:* Cytokine, Immunocompetent cells, Immunomodulator, Neem

### Introduction

Cancer is a disease that is very dangerous and deadly in the world. According to WHO data, cancer is the second leading cause of death in the world. In 2018 there were 18.1 million cases of cancer and 9.6 million deaths [1]. The incidence rate of cancer in Indonesia (136.2 / 100,000 population) ranks 8th in Southeast Asia, while in Asia, it is 23rd. For men, the highest incidence rate in Indonesia is lung cancer, 19.4 per 100,000, with average mortality reaching 10.9 per 100,000. Furthermore, liver cancer is in second place with a case rate of 12.4 per 100,000, while the average death rate reached 7.6 per 100,000. While the highest incidence rate for women is breast cancer, which is 42.1 per 100,000 population with an average

death rate of 17 per 100,000 population, followed by cervical cancer at 23.4 per 100,000 population with an average death rate of 13.9 per 100,000 population [2]. One of the cancer treatments so far is chemical therapy or chemotherapy. However, this method of treatment has disadvantages besides being large in cost and having side effects. Many kinds of research have been carried out for cancer treatment using various herbal medicines in recent decades. The ability of herbal medicines as anticancer is due to the presence of bioactive that function as antioxidants, anti-bacterial, anti-inflammatory, and immune modulatory. Immunomodulators are substances or drugs that can reverse the imbalance of the immu-

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ne system that is disturbed by stimulating and improving the function of the immune system.

Bioactive compounds in plants and fruits, named as phytochemicals in pharmacy, have been massively reported to exert protective activities against acute and chronic diseases in humans. The content of phenolic compounds in seaweed may reduce the risk of colon cancer [3]. *Hexagonia glabra* mushroom has potential as an anticancer agent and [4].

Another plant that has a positive effect on health is neem (*Azadirachta indica* Juss.). Neem is a tree whose trunk can reach 20 m. It has thick skin, slightly rough stems, even pinnate leaves, and an oval shape with jagged and pointed edges, while the fruit is a stone fruit with a length of 1 cm. Neem fruit is produced one to two times a year, is oval, when ripe the flesh is yellow, the seeds are covered with hard brown skin and inside are attached a white rind. The trunk is slightly bent and short; therefore, the wood is not large. The neem plant has several uses. In India, this plant is called the village pharmacy, where neem is used to cure skin diseases, anti-inflammatory, fever, antibacterial, antidiabetic, cardiovascular disease, and insecticides [5]. Health-promoting properties of the neem plant are widely discussed, in which these activities are attributed to the presence of bioactive compounds such as polyphenol and flavonoid [6]. Neem leaves contain several health-promoting compounds such as  $\beta$ -sitosterol, hyperoside, nimbolide, quercetin, quercitrin, rutin, azadirachtin, and nimbine. These compounds showed anticancer activity [7]. Numerous studies of neem bioactivities covered many topics, including antibacterial [3, 8], antioxidant [9, 10], anticancer [11], and immunomodulatory activities [12, 13].

Immunomodulation of the neem leaf extract occurred by altering the peritoneal macrophages population in experimental animals [13]. Research found that extract of neem leaves enabled to stimulation of expression of IFN $\gamma$  and components of immune surveillance cells (NK cell, T cytotoxic cell (CD8<sup>+</sup>), and macrophage) as observed in benzo(a)pyrene-induced mice [12].

To date, studies on immunomodulatory activity of methanolic extract from neem leaf focusing mainly on CD4<sup>+</sup>, CD8<sup>+</sup>, CD25<sup>+</sup>, and CD62L observed using flow cytometric analysis are scarcely reported. Therefore, this present work investigates the effect of methanolic extract of neem leaf as an immunomodulator on CD4<sup>+</sup>, CD8<sup>+</sup>, CD25<sup>+</sup>, and

CD62L cells.

## Material and Methods

### Experimental procedures

Twenty-five experimental animals (DDY mice, an average body weight of 200 g) were obtained from LPPT, Yogyakarta. Before the main experiment, acclimatization of the animals was carried out for seven days, in which they were fed and weighed daily during this period. The mice were then divided into five groups (comprising five animals in each group), i.e., group 1: negative control (normal), group 2: positive control (cancer), group 3: cancer-induced mice treated with neem leaf extract of 250 ppm, group 4: cancer-induced mice treated with neem leaf extract of 500 ppm, and group 5: cancer-induced mice treated with neem leaf extract of 1000 ppm.

Intraperitoneal injection of DMBA was carried out weekly for six weeks to the healthy mice (previously acclimatized for seven days) of groups 2, 3, 4, and 5, enabling to induce growth of cancer cells. The dose of DMBA injection is weight-independent. After confirming the cancer status by the presence of necrotic cells, the neem leaf extract was orally administered to mice at different levels (0 – 1000 ppm) for 14 days.

After completion of treatments, the animals were sacrificed, dissected from left dorsal to ventral. The spleen was collected and crushed with PBS (phosphate buffer solution) and then transferred into 15 ml-propylene tubes. The tubes were centrifuged at 2500 rpm (10°C, 5 min), then the supernatant was removed. The resuspension of the pellet was carried out using 1 mL of PBSA

### Flow Cytometric analysis

After incubation, cells were harvested and centrifuged at 2500 rpm (10°C, 5 min). Pellet was resuspended in 1 mL of PBS, transferred into four microtubes, and centrifuged, allowing for removal of the supernatant. The pellet was then stained using a combination of conjugated antibodies, i.e. (1) Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4, Phycoerythrin (PE)-conjugated rat anti-mouse CD25 and PE/ Cy5 conjugated rat anti-mouse CD62L; (2) FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD8; (3) FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse IFN $\gamma$  and PE/Cy5-conjugated rat anti-mouse TNF- $\alpha$  and (4) FITC-conjugated rat anti-mouse CD11B, PE-conjugated

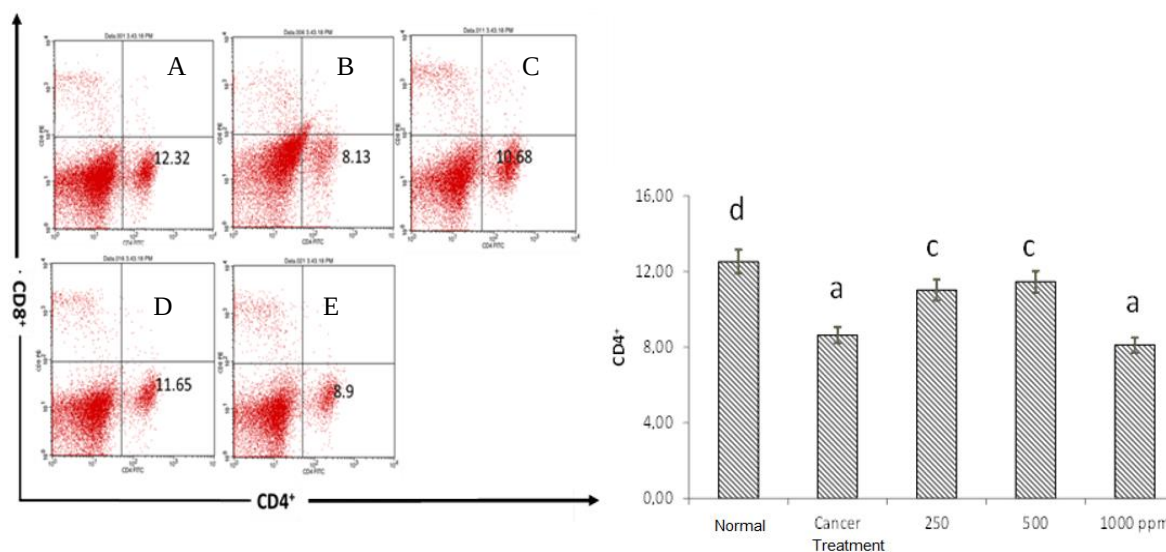


Figure 1. The relative number of CD4<sup>+</sup> cells in mice treated with different levels of neem leaves extracts. Note: (A) negative control/normal, (B) Dose/Cancer, (C) 250 ppm, (D) 500 ppm, and (E) 1000 ppm.

rat anti-mouse IFN $\gamma$ , PE/Cy5-conjugated rat anti-mouse NF- $\kappa$ B and APC-conjugated rat anti-mouse IL-6. The cells stained with the extracellular antibody were incubated for 20 min in an icebox. A fixative solution (50  $\mu$ L) of cytosol/cytoplasm was added and incubated for 20 min in the icebox. Alternatively, a rinse solution (500  $\mu$ L) was added and centrifuged. The supernatant was discarded, while the pellet was stained using an intracellular antibody and incubated for 20 min in an icebox. PBS (500  $\mu$ L) was added to both cell groups. Each sample was transferred into cuvet for flow cytometric analysis in the flow cytometer

#### Data analysis

Data obtained from flow cytometric analysis were evaluated using BD Cellquest Pro™ software, then statistically analyzed in SPSS version 21. Parametric analysis followed one-way ANOVA at a significance level of 5%. The significant difference was compared in the Duncan test.

#### Results and Discussions

The T helper cells, also recognized as CD4<sup>+</sup> cells, play a pivotal role in the immune system. Our experimental results showed that the treatments arranged demonstrated a significant difference in the number of CD4<sup>+</sup> cells. The cells in the cancer group tended to alleviate in comparison with those in the normal group. Meanwhile, mice treated with neem leaf extracts possessed a notice-

able increase in CD4<sup>+</sup> cells over cancer-induced mice. The reduced number of CD4<sup>+</sup> cells in cancer groups was mainly linked to protective activity against foreign disease and virus attacks. We found that the number of CD4<sup>+</sup> cells increased in mice treated with neem leaf extract, as exhibited in Figure 1.

This increment may relate to the role of bioactive compounds in neem leaf extract responsible for immunostimulatory, particularly activation of CD4<sup>+</sup> cells. Saponin and flavonoid are of foremost bioactive compounds in neem leaf extract, allowing to induce secretion of cytokinin associated with the activity of CD4<sup>+</sup> cells such as IFN $\gamma$  and IL2. The study showed that saponin could upregulate T helper cells by stimulating the number of IFN $\gamma$  [14]. The IFN $\gamma$  demonstrated a key role in the upregulation of MHC-II, thereby increasing the proliferation of CD4<sup>+</sup> cells. Besides, flavonoids served as immunostimulants by increasing the production of IL-2 [15]. IL2 could be induced by mitogens [16]. The research found that mitogen-activated protein kinases (MAPKs) regulate cell proliferation, allowing to catalyze the phosphorylation of cellular substrate that facilitates the proliferation of B cells and T cells [17].

#### CD8<sup>+</sup> cells in treated mice

The results showed that the administration of neem leaf extracts significantly altered the number of CD8<sup>+</sup> cells ( $p < 0.05$ ). Based on Figure 2, the

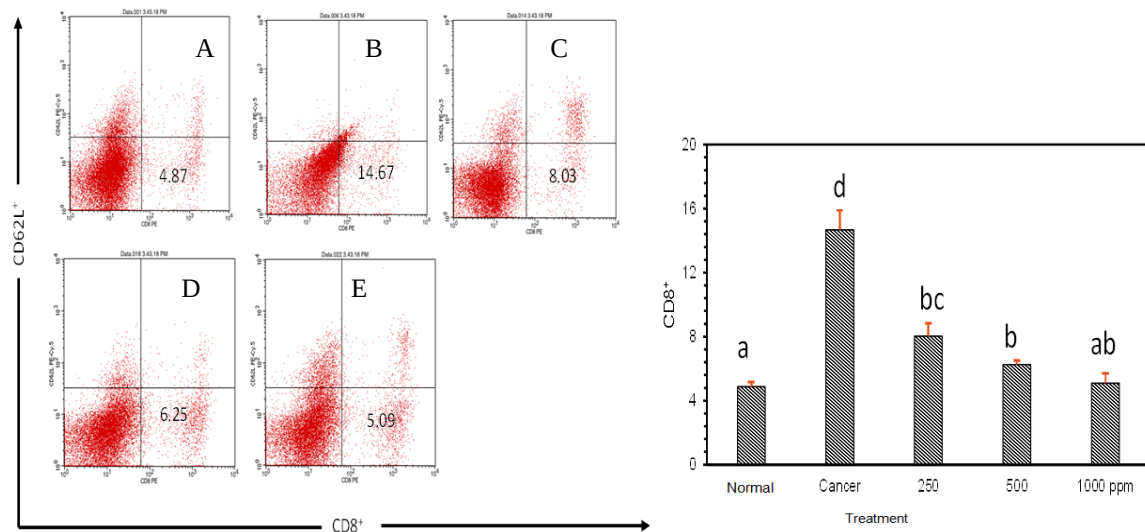


Figure 2. The relative number of CD8<sup>+</sup> cells in mice treated with different levels of neem leaf leaves extracts. Note: (A) negative control/normal, (B) Dose/Cancer, (C) 250 ppm, (D) 500 ppm, and (E) 1000 ppm.

relative number of CD8<sup>+</sup> cells was higher in cancer groups than in the normal group. Interestingly, a decrease of CD8<sup>+</sup> cells occurred in mice treated with neem leaf extract compared to cancer induced mice.

The number of CD8<sup>+</sup> cells tended to increase in cancer groups due to their role as suppressor T cells capable of reducing the development of cancer cells. In mice treated with neem leaf extract, the larger quantity of CD8<sup>+</sup> cells was ascribed to the role of saponin and flavonoid as active constituents, enabling modulation of the production of IL-2 in CD4<sup>+</sup> cells. The increase in IL-2 would alter the increment of CD8<sup>+</sup> cells. [18] found that IL-2 induced activation of CD8<sup>+</sup>, contributing to the synthesis of perforin and granzyme. Both constituents can degrade infected cells. The CD8<sup>+</sup> cells doubled several times as a response to the infections [19].

#### **The effect of neem leaf extract on CD62L**

The naive T cells (CD4<sup>+</sup>CD62L<sup>+</sup>) constitute mature lymphocyte cells that migrate from the thymus, are not differentiated, and are exposed to their specific antigen. The activation of T cells leads them to the process of differentiation into effector T cells and memory cells, followed by their migration into lymphoid and non-lymphoid tissues [20]. In this work, the administration of neem leaf extract promoted a significant difference in the amount of CD4<sup>+</sup>CD62L<sup>+</sup>. As shown in Figure 3, there was a decrease in the relative number of CD4<sup>+</sup>CD62L<sup>+</sup> in cancer mice and neem treatment.

The reduced amount of CD4<sup>+</sup>CD62L<sup>+</sup> was associated with activation of CD4<sup>+</sup>CD62L<sup>+</sup> previously existed as naive cells, induced by the presence of cancer cells. [18] asserted that CD62L constituted a marker for cell activation; thus, the reduced number of CD62L T cells could indicate activation of naive T cells into CD4<sup>+</sup>. The decreasing expression of CD62L in immature T cells (CD4<sup>+</sup>CD62L<sup>+</sup>) suggests that T cells require CD62L to attach and roll on the endothelial cells in blood vessels. The CD62L expression was very high in naive T cells but lower in activated T cells. It modulated the migration of naive T cells into lymph nodes in which antigen and immune response initiation existed [21]. The expression of CD62L resulted from TCD4<sup>+</sup> proliferation and occurred as a response to stimulation from antigen [22].

The number of CD4<sup>+</sup>CD62L<sup>+</sup> cells increased due to stimulation induced by flavonoids present in the neem leaf extract. The increasing amount of naive T cells and memory cells in the infectious group is closely related to the mitogen of neem leaf extract. The mitogen originating from plants enhanced the immune system's proliferation and differentiation of T and B cells. The alteration of the relative number of CD4<sup>+</sup>CD62L<sup>+</sup> in treated mice than in the normal group caused by neem leaf extract was exhibited in Figure 3.

#### **The effect of neem leaf extract on CD25<sup>+</sup>**

Based on statistical analysis, neem leaf extract had a significant effect on CD25<sup>+</sup> at the  $\alpha$  5% significance level. Our experiment showed an increa

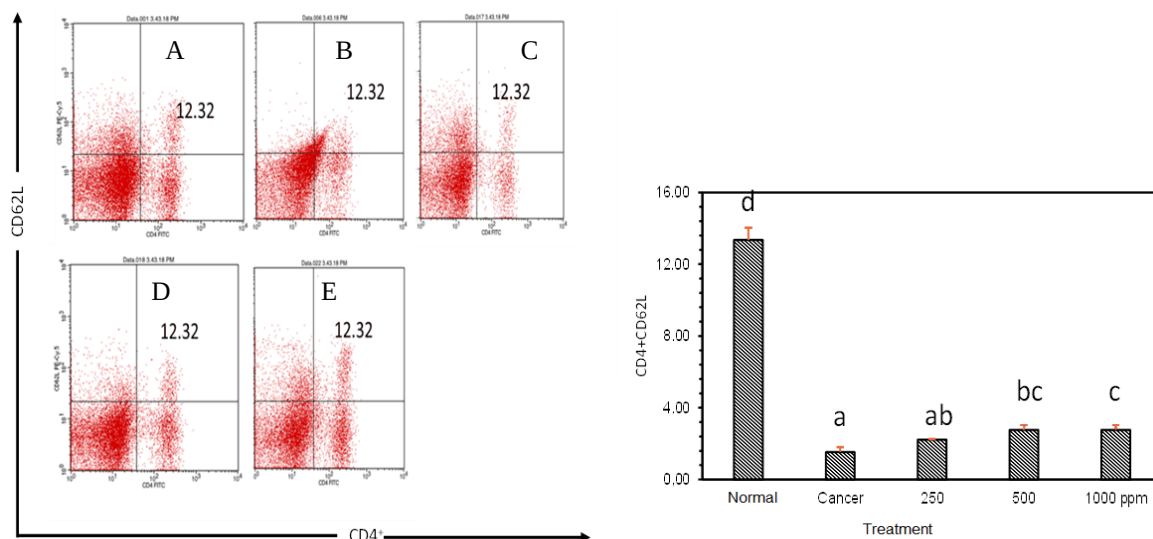


Figure 3. The relative number of CD4+CD62L+ cells in mice treated with different levels of neem leaves extracts. Note: (A) negative control/normal, (B) Dose/Cancer, (C) 250 ppm, (D) 500 ppm, and (E) 1000 ppm.

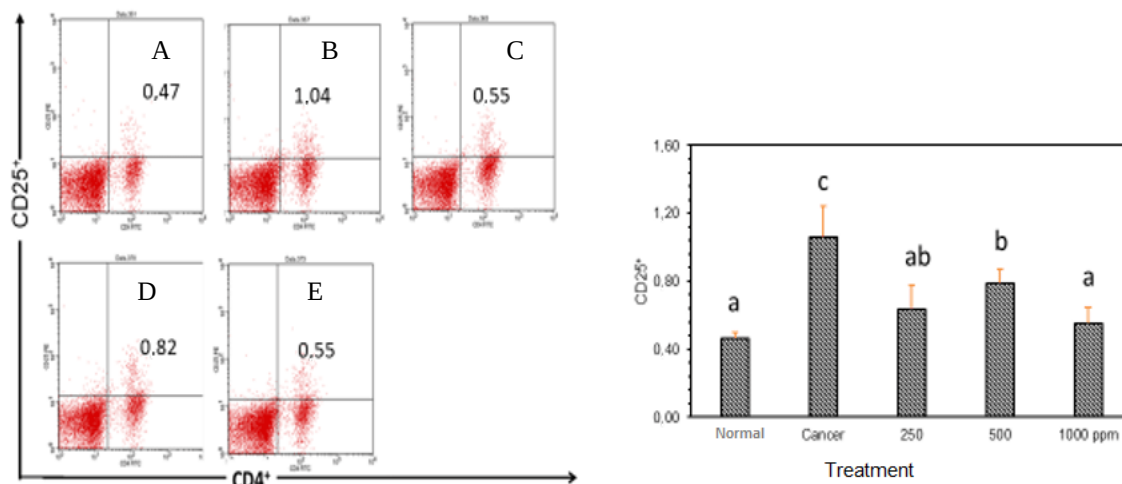


Figure 4. The relative number of CD25+ cells in mice treated with different levels of neem leaf extracts. Note: (A) negative control/normal, (B) Dose/Cancer, (C) 250 ppm, (D) 500 ppm, and (E) 1000 ppm.

se of CD25<sup>+</sup> in cancer mice, but a profound decrease in CD25<sup>+</sup> was observed in mice treated with neem leaf extract of 500 ppm compared with normal mice, as depicted in Figure 4.

CD25<sup>+</sup> is a transmembrane glycoprotein and component for the receptor of IL-2. Our experiment showed an increase of CD25<sup>+</sup> in cancer mice, but a profound decrease in CD25<sup>+</sup> was observed in mice treated with neem leaf extract of 500 ppm compared with normal mice, as depicted in Figure 4. The significant increase of CD25<sup>+</sup> was associated with saponin and flavonoids present in the neem leaf extract. The saponin and flavonoid

could stimulate the response of cellular immune through rising production of IL-2 and IFN $\gamma$ . The rise of IL-2 da Figure 4. The relative number of CD25<sup>+</sup> cells in mice treated with different levels of neem leaf extracts n IFN $\gamma$  indirectly promoted CD4<sup>+</sup> T cell precursor differentiation to CD4<sup>+</sup>CD25<sup>+</sup> T cells.

**The effect of neem leaf extract on IL-10 Cell expression in mice**

Statistically, the significant impact of neem leaf extract on the number of IL-10 was observed in normal and treated mice. Figure 5 shows the

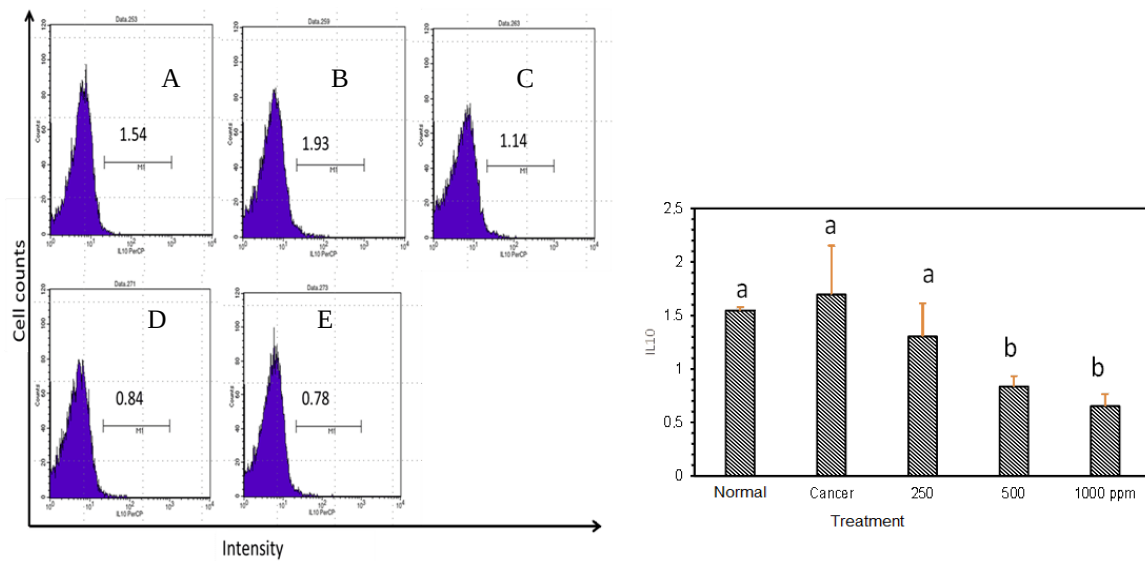


Figure 5. The relative number of IL-10 cells in mice treated with different levels of neem leaves extracts. Note: (A) negative control/normal, (B) Dose/Cancer, (C) 250 ppm, (D) 500 ppm, and (E) 1000 ppm.

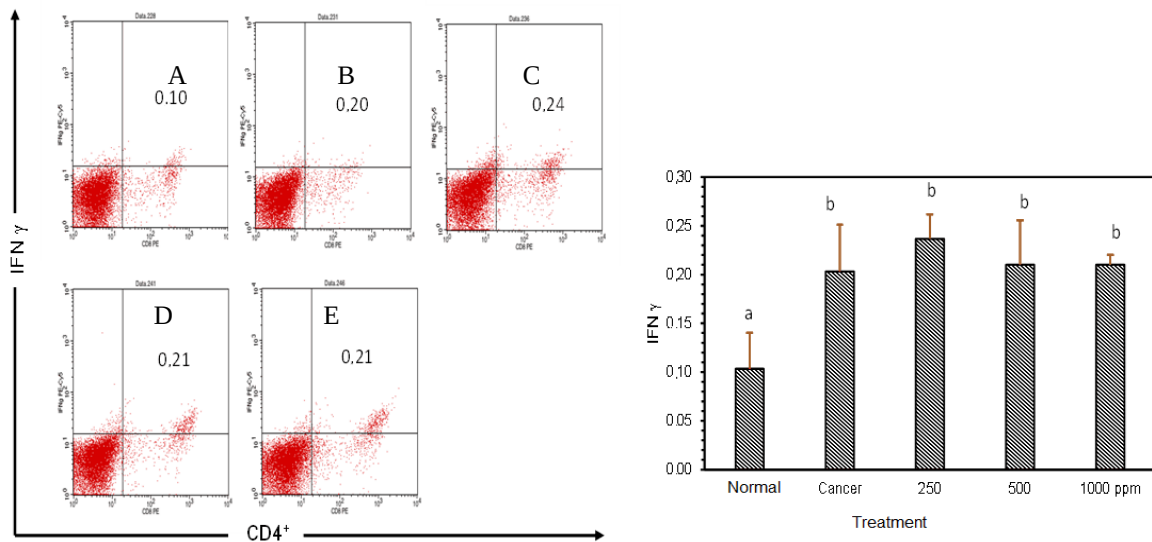


Figure 6. The relative number of IL-17 cells in mice treated with different levels of neem leaf extracts. Note: (A) negative control/normal, (B) Dose/Cancer, (C) 250 ppm, (D) 500 ppm, and (E) 1000 ppm.

effect of neem leaf extract on IL10. The more neem leaf extract that is used causes the amount of IL-10 to decrease.

Interleukin 10 (IL-10) exhibited the most potent anti-inflammatory cytokine, serving as a central response against pathogens, protecting hosts, as well as regulating homeostasis in normal tissues [23]. The polyphenol exerted several promising bioactivities such as antioxidants, anti-inflammation, and anticancer. The mode action of anticancer properties by polyphenol is based on blocking IL-10 secretion and sensor for IL-12 secretion [24]. IL-12 modulates the secretion of IFN $\gamma$  [25].

The effect of neem leaf extract on the expression of IL-10 was presented in Figure 5.

**The effect of neem leaf extract on IL-17 Cel expression in mice**

Interleukin-17 (IL-17) is a pro-inflammatory cytokine and is generated by Th17 [26]. The results showed that the relative number of IL-17 cells significantly differed between normal and cancer and treated mice.

As depicted in Figure 6, the number of IL-17 cells in cancer mice was higher than that in normal mice, while treated mice showed the lowest num-

ber of IL-17. The higher number of IL-17 cells in cancer mice resulted from inflammation. However, treatment of neem leaf extracts could suppress the expression of IL-17, enabling to inhibit of inflammation and reduce transcription of Th1 and Th17.

## Conclusion

The study demonstrated that neem leaf (*Azadirachta indica* Juss) extract has immunomodulatory activities, especially at the concentration of 500 ppm. Neem leaf extract affects increasing cell T CD4<sup>+</sup> and CD8<sup>+</sup>, pressure anti-inflammatory cytokine IL10, and decreasing pro-inflammatory cytokine IL17.

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