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Research Report

## EFFECT OF AFRICAN LEAF (VERNONIA AMYGDALINA) TO IL-6 AND IL-10 LEVEL ON STAPHYLOCOCCUS AUREUS INFECTION

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### ABSTRACT

Currently, infectious disease is increase in world wide. The African leaf (*Vernonia amygdalina*) – VA is used to antimicrobial treatment. It may protect the host against microbial attack in several ways. This plant has attracted the interest of researchers in recent decades because of the constituents have important roles in modulating immune system in bacteria infection. The aim of study is to analyze the prophylactic activity of VA's ethanol extract in modulating the levels of IL-6 and IL-10 as well as the number of bacteria in male Wistar rats that were (*Staphylococcus aureus*) – SA – infected. There were as many as 30 rats were divided into 5 treatment groups: negative control (NC) was treated by CMC Na 2% (w/v); positive control (PC) was treated by 9mg/200g body weight (BW) of cephadroxil; T1; T2; and T3 were respectively treated with ethanol extract of VA of doses 20mg/200g BW; 40mg/200g BW and 80mg/200g BW. After the oral treatment was administered, all the rats were infected with 0.25mL ( $3 \times 10^8$ cfu) SA via intra peritoneal route. Their blood was drawn in order to identify the IL-6 and IL-10 levels by ELISA. Furthermore, their peritoneal fluid was also taken to count the number of survived bacteria by pour plate method. The results are showed median of IL-6 and IL-10 levels as well as bacterial number respectively in NC 370.530pg/mL; 67.044pg/mL;  $7.4 \times 10^3$ cfu/mL; in PC 234.556pg/mL; 42.839pg/mL;  $6.8 \times 10^3$ cfu/mL; in T1 164.019pg/mL; 17.240pg/mL;  $1.1 \times 10^4$ cfu/mL; in T2 49.291pg/mL; 2.961 pg/mL;  $6.3 \times 10^3$ cfu/mL and in T3 43.342pg/mL; 13.235pg/mL;  $7.1 \times 10^3$ cfu/mL. These results are implied that VA's ethanol extract is effective as a prophylactic agent to suppress the bacterial invasion at dose of 40mg/200g BW in Wistar rat particularly shown by the decrease level of IL-6 and the number of bacteria.

**Keywords:** *Vernonia amygdalina*, IL-6, IL-10, bacterial number, *Staphylococcus aureus*.

### ABSTRAK

Saat ini, penyakit infeksi meningkat di dunia. Daun Afrika (*Vernonia amygdalina*) – VA digunakan sebagai terapi antimikroba. VA dapat melindungi host terhadap serangan mikroba melalui berbagai macam cara. Tanaman ini menarik perhatian para peneliti pada dekade terakhir karena VA memiliki berbagai kandungan yang berperan penting dalam memodulasi sistem imun pada infeksi bakteri. Pada penelitian ini, dilakukan analisa terhadap kemampuan profilaksis dari ekstrak etanol VA dalam memodulasi kadar IL-6 dan IL-10 serta jumlah bakteri pada tikus Wistar jantan yang terinfeksi (*Staphylococcus aureus*) – SA. Tikus Wistar sebanyak 30 ekor dibagi ke dalam 5 kelompok uji yaitu kontrol negatif (NC) yang diterapi dengan CMC Na 2% (b/v), kontrol positif (PC) yang diterapi sefadroksil 9mg/200g BB, kelompok T1, T2 dan T3 yang diterapi dengan ekstrak etanol VA dengan dosis: masing-masing 20mg/200g BB; 40mg/200g BB; dan 80mg/200g BB, secara berurutan. Setelah diberikan terapi, tikus diinfeksi dengan bakteri SA sebanyak 0,25mL ( $3 \times 10^8$ cfu) secara intra peritoneal. Darah tikus dikoleksi untuk dianalisa kadar IL-6 dan IL-10 menggunakan ELISA dan dikoleksi pula cairan peritoneal untuk dihitung jumlah bakteri yang bertahan hidup menggunakan metode tuang. Hasil median dari kadar IL-6, IL-10 dan jumlah bakteri secara berurutan adalah sebagai berikut: pada kontrol negatif (NC) 370,530pg/mL; 67,044pg/mL;  $7,4 \times 10^3$ cfu/mL; pada kontrol positif (PC) 234,556pg/mL; 42,839pg/mL;  $6,8 \times 10^3$ cfu/mL; pada T1 164,019pg/mL; 17,240pg/mL;  $1,1 \times 10^4$ cfu/mL; pada T2 49,291pg/mL; 2,961 pg/mL;  $6,3 \times 10^3$ cfu/mL; pada 43,342pg/mL; 13,235pg/mL;  $7,1 \times 10^3$ cfu/mL. Hasil penelitian ini mengimplikasikan

bahwa ekstrak etanol VA efektif sebagai senyawa profilaksis yang mencegah invasi bakteri, khususnya pada dosis 40mg/200g BB tikus karena pada dosis tersebut terjadi penurunan kadar IL-6 dan jumlah bakteri.

**Kata kunci:** *Vernonia amygdalina*, kadar IL-6, kadar IL-10, jumlah bakteri, *Staphylococcus aureus*.

## INTRODUCTION

Globally, there is an increase in infectious disease especially in bacteria infection. It was caused of host body defense mechanism can't control the immune system.<sup>1</sup> Therefore, it is an evident from human history that we have to some new product to modulate immune system against bacteria infection. One of which is medicinal plants that it have been utilized as therapeutic agents in variety of disease including infection diseases. Medicinal plants are through to be mediated through inhibition and modulating cell-signaling pathways in immune system.<sup>2</sup> The immunomodulating characteristic of medicinal plants is safety, effectiveness, minor side effect and cultural acceptability.<sup>3</sup>

*Vernonia amygdalina* (VA) is one of medicinal plants<sup>4</sup> and a member of the Asteraceae family. This plant is a small tree in 2-5 m of size. *Vernonia amygdalina* leaf is ellipse in form and about 6 mm of diameter. The green leaves are showing bitter taste and odor characteristics.<sup>5</sup> This bitter taste serves as a protection of animals attack such as insects and microbes.<sup>6</sup> Bitter taste is due to the flavonoids and sesquiterpenes lactones contained in VA.<sup>7</sup> *Vernonia amygdalina* contains many other constituents such as: tannins,<sup>8</sup> saponins, alkaloids, terpenoids, stigmastane-type steroid glycosides, coumarin, phenolic acids, lignans, xanthenes, anthraquinones and edotides.<sup>9</sup> Several constituents of VA that have been reported to function as antimicrobial agent and modulate the immune system is known as luteolin<sup>5</sup> and myricetin; andrographolide;<sup>9</sup> and chlorogenic acid.<sup>10</sup>

Previous studies were illustrated the antimicrobial effects of VA in ethanol extract. This ethanol extract is more effective to exhibit antimicrobial effects than water extract.<sup>4,11-13</sup> Previous in vitro studies is proved that ethanol extracts of VA showed high potency and effectivity against *Staphylococcus aureus* (SA).<sup>11,13</sup>

*Staphylococcus aureus* is a pathogenic bacterium<sup>14</sup> that belong to gram-positive bacteria with coccid (round) form and also known as a facultative anaerobes. It has a complex cell wall consisting of murein, teichoic acids and surface proteins.<sup>15</sup>

In the human body, lipoteichoic acid (LTA) on the surface of SA's cell wall was known as Pathogen Associated Molecular Patterns (PAMPs) which generally be recognized by Pattern Recognition Receptors (PRRs) such as Toll Like Receptors (TLRs)-1/2 or TLRs-2/6. The PRRs is

the property of the immune cells such as macrophages, dendritic cells, endothelial cells, mast cells, eosinophils and B cells.<sup>16,17</sup> PRR will exhibit a signal that may attract the Nuclear Factor kappa B (NFκB) transcription factor to enter the nucleus and synthesize pro-inflammatory cytokines such as interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α, IL-6,<sup>18</sup> IL-12 and IL-8 or CXCL8.<sup>19</sup>

IL-6 is known as a pleiotropic cytokines found in each organ system. It is synthesized by mononuclear phagocytes, dendritic cells, vascular endothelial cells, fibroblasts and other cells in response to PAMPs and IL-1 and TNF stimulation.<sup>17</sup> IL-6 production immediately increases in the acute inflammatory condition that occurs due to infection, injury, trauma and other stress conditions. These cytokines retain extracellular and intracellular growth from SA but excessive production can lead to systemic inflammation with damaging effects rather than protection of the host.<sup>18</sup> In order to balance the IL-6 effects, another cytokine, IL-10 is produced and is served as an anti-inflammatory agent. This cytokine is produced by immune cells including macrophages and active dendritic cells, T regulators (Tregs), T Helper (T<sub>H</sub>)-1, and T<sub>H</sub>2 cells. IL-10 is also produced by several B lymphocytes which show immune suppression function, called regulatory B cells. IL-10 is also an important cytokine that regulate the immune responses in infection diseases.<sup>20</sup>

In infection caused by SA, generally, immune cells are stimulated to promote pro-inflammatory cytokine (such as: IL-6) dan anti-inflammatory cytokine, such as IL-10.<sup>21</sup> However, until recently, there is no study identify ethanol extract of VA activity in modulating these cytokines in microbial infections. Therefore, this study was conducted in vivo to study the VA's ethanol extract activities in immune system in Wistar rats which infected by SA. Further, this study is aimed to analyze prophylactic activity of VA's ethanol extract in modulating levels of IL-6, IL-10 as well as the decrease of the number of bacteria in male wistar rats that were SA-infected.

## MATERIAL AND METHOD

### I. Collection of Plant Materials

Fresh leaves of *Vernonia amygdalina* were obtained from Jember, East Java, Indonesia. The leaves were dried in an oven at 60°C and then blended into powder.

## II. Preparation of Ethanol Extract

*Vernonia amygdalina* powder was macerated with 96% ethanol (1: 5) for 72 hours was followed by filtration with Whatman No.1 filter paper and was evaporated in a rotary evaporator, the thick extract was yielded. The thick extract dissolved in CMC Na 2%.

## III. Standardization and Phytochemistry Screening Standardization of the Extract

### Organoleptic

Organoleptic examination includes examination of color, odor, and taste.<sup>22</sup>

### Total ash content

Thickened extracts were weighed 2 to 3 grams and were placed into the incandescent and were tared silicate crucible and were heated in furnace at 600°C for 3 hours. Then, it was cooled and weighed. If charcoal stays, hot water was added, then was stirred and was filtered with ash-free filter paper. The filtering residue and filter paper were applied to the same crucible. The filtrate was added to the crucible, evaporated and incandescent until the weight was fixed, then weighed. Total ash content was calculated against the weight of thick extract expressed in % b/b.<sup>23</sup>

### Acid insoluble ash content

The ash was boiled with 25mL of diluted sulfuric acid for 5 minutes, was collected parts which were not soluble in acid, filtered through glassy crust or ash-free filter paper, was washed with hot water, incandescent until the weight remained, then was weighed. Then the ash content which is insoluble in acid against thick extract was calculated in % b/b.<sup>23</sup>

### Loss on drying

The dried-shrinkage method was determined as follows: Carefully weigh 1 to 2 grams of substances which have previously been heated at 105°C for 30 minutes and have been tared. Before weighing, the extract was flattened in a bottle and then put into a drying chamber and dried at 105°C to a fixed weight. Let the bottle be closed and cooled in the desiccator to room temperature. Then note the fixed weight obtained to calculate the percentage of drying loss.<sup>23</sup>

### Phytochemistry

The phytochemical screening of dried simplicia and ethanol extract of VA: alkaloids, flavonoids, saponins, tannins was analyzed using the standard methods as described by Soetarno (2008);<sup>24</sup> steroids and terpenoids, antraquinones by Kristanti et al (2008);<sup>25</sup> phenol and glycoside by Harbourne (2008).<sup>26</sup>

## IV. Collection of *Staphylococcus aureus*

Isolate of *Staphylococcus aureus* ATCC 25923 from Department of Microbiology, Widya Mandala Catholic University. These bacteria were rejuvenated in nutrient broth medium and were incubated at 37°C for 24 hours. Then the isolate was mixed in 0.9% NaCl and standardized to McFarland IV (1.2x10<sup>9</sup>cfu/mL).

## V. Determination of Antimicrobial effect

Thirty male wistar rats were divided into 5 groups :

Negative Control (NC): rats were treated CMC Na 2%

Positive Control (PC): rats were treated 9mg/200g BW of cefadroxyl antibiotics

T1: rats were treated 20mg/200g BW of *Vernonia amygdalina* ethanol extract

T2: rats were treated 40mg/200g BW of *Vernonia amygdalina* ethanol extract

T3: rats were treated 80mg/200g BW of *Vernonia amygdalina* ethanol extract

The rat in both NC, T1, T2 and T3 groups were treated with 1 mL VA ethanol extract orally 3 times a day. The next day, the PC group was administered 1mL of cefadroxyl orally. Then all rats were injected with 0.25mL SA suspension in 0.9% NaCl (3x10<sup>8</sup>cfu) intraperitoneally. After 24 hours of bacteria injection, all rats were sacrificed and blood was withdrawn intracardially to measure IL-6 and IL-10 levels. The peritoneal fluid was collected to count the number of survived bacteria.

## VI. ELISA

Elabscience Rat IL-6 ELISA KIT (Catalog No. E-EL-R0015) and Elabscience Rat IL-10 ELISA KIT (Catalog No. E-EL-R0016) were used to quantify the IL-6 and IL-10.

## VII. Bacterial Counting

The fluid from peritoneal rat was identified by pour plate method. The 0.1mL peritoneal fluid was withdrawn and was placed in a tube containing 9.9mL of sterile aquadest (tube 1). Then, 1mL from the tube 1 was taken and it into 9mL sterile aquadest (tube 2) and so on until tube 3 (twice replication was performed). After that, from each tube, 1mL was taken and placed in a petri dish, which subsequently was added 10mL of nutrients sterile agar (at 50°C) and was rotated in order to obtain to mixture of bacteria. All petri dish were incubated at 37°C for 24 hours. Afterwards, the number of colonies was calculated.<sup>27</sup>

## RESULT AND DISCUSSION

The standardization results were showed that VA's ethanol extract (Table 1) have a dark green color, characteristic odor and bitter taste. The total ash content shows high mineral content such as calcium, chlorine, chromium, copper, iron, potassium, magnesium, manganese, nickel, phosphorus, potassium, sodium in this plant<sup>28,29</sup> while the acid insoluble ash content was showed the contamination of fine particles from sand and soil from the environment.<sup>30</sup> Furthermore, 88.36% of the compounds lost during the drying process.<sup>22</sup>

Phytochemical screening was indicated that both simplicia and ethanol extract of VA contained flavonoids, saponins, tannins, steroids, terpenoids, phenols and

glycosides, while alkaloids and anthraquinones was not detected (Table 2). This might be due to geographical differences where the plants grow.

In this study, it was observed the effectiveness of VA in modulating immune system in Wistar rat that was infected by SA. Generally, in infectious condition induced by bacteria, the body generates a defence mechanism in reaction to the encountered microbes or their products. The defence is preceded by the presence of immune cells such as macrophages, dendritic cells, neutrophils, natural killer cells, and limfoid cells. They were resolved the microbes through two main actions: the first is recruiting phagocytes and other leukocytes to destroy the microbes (indicates inflammatory reaction) and secondly by limiting microbial replication or killing microbial-infected cells without inflammatory reaction.<sup>17</sup>

During inflammation process, immune cells recognized the molecular structure produced by SA through a binding between TLRs-1/2 or TLRs-2/6 with lipoteichoic acid (LTA). This binding was activated the transcription factor, NFκB, to produce high amounts of IL-6 levels.<sup>16-18</sup> This cytokine was needed when inflammation occurs to increase the formation of neutrophils in bone marrow and recruitment of neutrophils to the site of infection to replace the leukocyte cells that died during inflammation.<sup>17</sup> However, high levels of IL-6 also was stimulated a negative impact which is correlated with disease progression<sup>31</sup> and is contributed in exacerbating inflammation so it triggered to autoimmune diseases.<sup>17</sup> Therefore, the effectivity of the VA's ethanol extract in reducing production of IL-6 levels (Figure 1) and may be potential to reduce inflammation.

The VA's constituents that are playing role as the IL-6 reducing agents are luteolin and myricetin, they are belongs

to the flavonoid groups. Luteolin inhibits NF-κB activation by blocking the degradation of IκBα and phosphorylation of p65.<sup>18</sup> Whereas, myricetin works by inhibiting the activation of p38 and extracellular signals from TLR2/6 and also blocking the degradation of IκB. Myricetin which given as prophylactic agents can significantly reduce IκB degradation,<sup>32</sup> furthermore, Viljoen et al. (2016) were reported that myricetin also inhibits the activation of ERK-1/2, AKT and p38 induced by LTA,<sup>33</sup> thus these mechanisms were blocked the production of pro-inflammatory cytokines, IL-6.<sup>32</sup>

The other constituents of VA ethanol extract may exhibit molecular function in regards of IL-6 reduction such as andrographolide that belongs to terpenoids groups. Andrographolide also plays a role in decreasing IL-6 by inhibiting NFκB activation, suppressing iNOS, and preventing oxygen radicals produced by neutrophils.<sup>34</sup> Furthermore, tannin, that is one of VA's constituents has ability to reduce intracellular kinase phosphorylation and inhibit NFκB at p65 and its catalytic activity, therefore those processes may decrease the IL-6 levels.<sup>35</sup>

Moreover, chlorogenic acid in VA's ethanol extract belongs to phenol group suppresses the expression of the NFκB signaling pathway inhibits the activation of this signaling pathway and reducing inflammatory cytokines production. A previous study was confirmed that taking chlorogenic acid can reduce levels of NFκB p50 and IKKα/β.<sup>36</sup>

On the other hands, in presence of microbial infections, immune cells were also produced anti-inflammatory cytokines such as IL-10 to reduce inflammation.<sup>18</sup> In

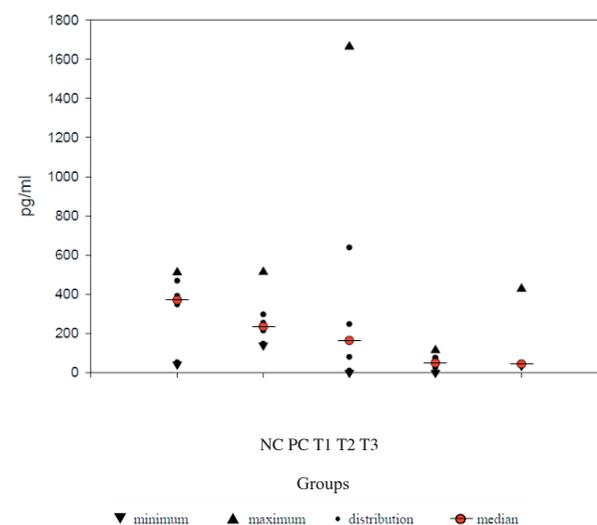
**Table 1.** Standardization Results of *Vernonia Amygdalina*

Determination	Ethanol extract
Organoleptics	Color: dark green Odor: characteristic Taste: bitter
Total ash content	16.18 ± 0.48%
Acid insoluble ash content	0.822 ± 0.18%
Loss on drying	88.36 ± 0.74%

**Table 2.** Phytochemical Screening of *Vernonia amygdalina*

Determination	Simplicia	Ethanol extract
Alkaloids	-	-
Flavonoids	+	+
Saponin	+	+
Tannin	+	+
Steroid	+	+
Terpenoids	+	+
Anthraquinone	-	-
Phenol	+	+
Glycoside	+	+

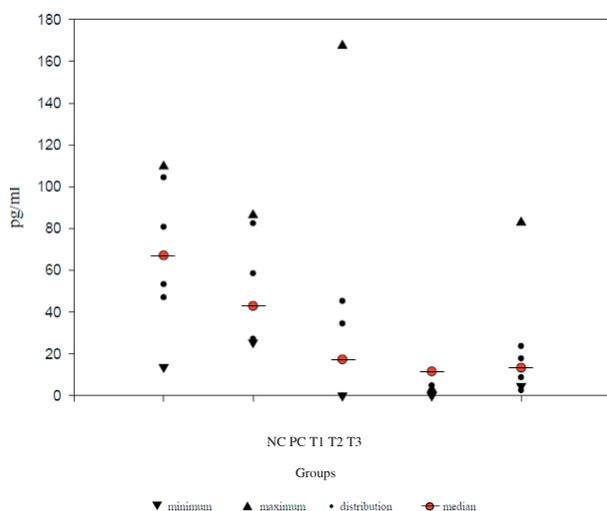
Description: (+) = Identified and (-) = not identified



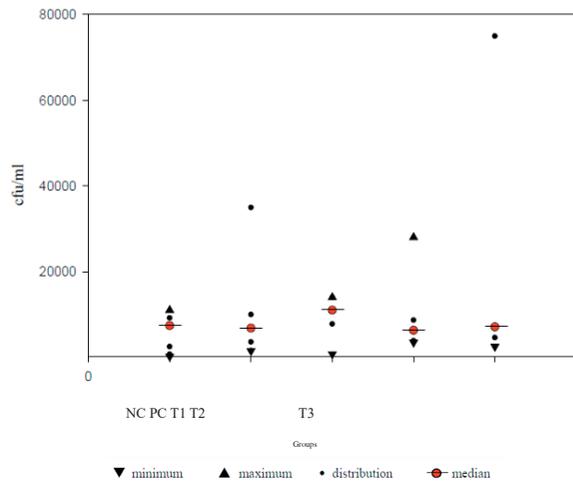
**Figure 1.** Graphic of IL-6 Levels Distribution. NC: Given CMC Na 2%; PC: Given 9mg/200g BW of Cefadroxyl Antibiotics; T1: Given 0mg/200g BW of *Vernonia Amygdalina* Ethanol Extract; T2: Given 40mg/200g BW of *Vernonia Amygdalina* Ethanol Extract; T3: Given 80mg/200g BW of *Vernonia Amygdalina* Ethanol Extract.

contrast, this study was found that IL-10 levels decreased after VA's ethanol extract administration (Figure 2). This might be occurred due to the myricetin and chlorogenic acid which were suppressed the expression of JAK/STAT signaling pathways<sup>34</sup> and thus were inhibited the production of IL-10 in Wistar rats's immune cells.<sup>17</sup>

Cheng and Iyer (2012) were reported that the majority of intracellular infections were better controlled or were cleaned quickly in a no IL-10 state. Decreasing IL-10 signaling leads to increase host survival after infection and to increase adaptive immune response, including CD4<sup>+</sup> T cells that produce Interferon (IFN)- $\gamma$ .<sup>37</sup> Similar to Cheng and Iyer's report, Riley et al. (2008) were observed that IL-10 is an important regulator component in almost all infections.<sup>20</sup> This statement is clarified through a research conducted by McLoughlin et al. (2017), that during systemic acute infection was induced by SA, IL-10 was regulated local and systemic proinflammatory responses that prevented the host from immunopathology condition caused by bacteria spreading.<sup>38</sup> In infections which were caused by SA, the decreased of IL-10 levels can increase IFN- $\gamma$ ,<sup>39</sup> IL-17, IL-22 and CXCL1. Therefore it was stimulated the increasement of T<sub>H</sub>1 cells as well as activated the phagocytes to clear the bacteria.<sup>38</sup> In addition, the IL-10 decreased levels may increase the expression of costimulators and Major Histocompatibility Complex (MHC) II molecules and IL-12 production in macrophages and dendritic cells. IL-12 is the main cytokine that stimulate adaptive immune response, T<sub>H</sub>1 cells, which will secrete IFN- $\gamma$ . IFN- $\gamma$  plays an important role in the reaction of innate and adaptive immune cells against intracellular microbes.<sup>17</sup> Therefore, IL-10 deficiency in intracellular infections can reduce the



**Figure 2.** Graphic of IL-10 Levels Distribution. NC: given CMC Na 2%; PC: Given 9mg/200g BW of Cefadroxyl Antibiotics; T1: Given 0mg/200g BW of *Vernonia Amygdalina* Ethanol Extract; T2: Given 40mg/200g BW of *Vernonia Amygdalina* Ethanol Extract; T3: Given 80mg/200g BW of *Vernonia Amygdalina* Ethanol Extract.



**Figure 3.** Graphic of Bacterial Numbers Distribution. NC: Given CMC Na 2%; PC: Given 9mg/200g BW of Cefadroxyl Antibiotics; T1: Given 0mg/200g BW of *Vernonia Amygdalina* Ethanol Extract; T2: Given 40mg/200g BW of *Vernonia Amygdalina* Ethanol Extract; T3: Given 80mg/200g BW of *Vernonia Amygdalina* Ethanol Extract. The Maximum Value in T1 group is 210000 and The Maximum Value in T4 Group is 1300000

number of microbes by activating the adaptive immunity to kill bacteria.<sup>38</sup>

Moreover, Figure 3 shown that VA's ethanol extract also plays a role in reducing the number of bacteria. This extract occurred due to the content of andrographolide and luteolin. Andrographolide had a bacteriostatic effect. Andrographolide will weaken DNA synthesis of SA so it will produced inhibition on biosynthesis pathway of intracellular DNA in *Staphylococcus aureus*.<sup>40</sup> In addition, luteolin also had antibacterial effects by inhibiting the activity of *Staphylococcus aureus* bacteria in DNA topoisomerase I and II which will result in a decrease in nucleic acid and protein synthesis.<sup>41</sup>

## CONCLUSION

This study was indicated that the optimum dose of VA's ethanol extract in exhibiting IL-6 and IL-10 modulation in Wistar rats is 40mg/200g BW. This dose can decreased IL-6 levels and bacterial numbers which tent to will decrease the inflammation. It may imply the effectivity of this plant as a prophylactic agent to prevent SA infection.

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