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Immunomodulator effect of *Cnidoscolus aconitifolius* leaves extract on $CD4^+$ and $CD8^+$ expression in *Salmonella typhimurium*-infected mice

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ABSTRACT

Typhoid fever is a common health problem in the community caused by *Salmonella* bacteria. The incidence rate of this infection will increase if a person's immune system is weakened. Plant extracts have been widely studied for their role in various pharmacological effects, including immunomodulatory effects. Among the plants with the potential to be used as an immunomodulatory substance is *Cnidoscolus aconitifolius*. *Cnidoscolus aconitifolius* leaves extract (CAE) contains flavonoids related to immunomodulatory activity. This study intends to ascertain how administering CAE affects the expression of $CD4^+$ and $CD8^+$ in Babl/c mice that have been infected with *Salmonella typhimurium* bacteria. The study was started by preparing 70% ethanol extract from *Cnidoscolus aconitifolius* leaves. Immunomodulatory activity testing was carried out preparing 30 Babl/C mice as experimental animals. Six mouse groups (the treatment group, the negative control, the positive control, and the healthy control) were allocated at random by giving CAE doses of 100 mg/kgBW, CAE doses of 200 mg/kgBW, and CAE doses of 400 mg/kgBW. Induction was carried out by oral infection with *Salmonella thypimurium* bacteria. After 3 days the infected mice were treated orally once a day for 7 days. Evaluation of $CD4^+$ and $CD8^+$ expression was carried out using the flow cytometry method of the lymph organs. The data was analyzed using the anova test and then the SPSS for Windows tool was used to do the post hoc test (Tukey). The results showed that giving CAE at doses of 100 mg/kgBW, CAE doses of 200 mg/kgBW and CAE doses of 400 mg/kgBW could increase the expression ratio of $CD4^+$ and $CD8^+$. Conversely, administering 400 mg/kgBW of CAE produced noticeably different outcomes ($p<0.05$) from the negative control. This shows that the CAE has potential as an immunomodulatory agent that can improve immune function.

Keywords: *Cnidoscolus aconitifolius*, immunomodulator, *Salmonella typhimurium*, $CD4^+$, $CD8^+$

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INTRODUCTION

Infectious diseases are currently the second leading cause of death worldwide in the past ten years, after cardiovascular sickness. The sickness is usually caused by a variety of pathogens, including bacteria, worms, viruses, protozoa, and parasitic fungi that develop on the surface of the body or infect it. Typoid dementia is the infection with the highest incidence in some Asian countries (Fitrya et al., 2020). *Salmonella enterica* infection is the main cause of acute fever, where *Salmonella typhimurium* derivatives are the most abundant (Cordero-Alba et al., 2016). Someone who has a low immune system will be very at risk for contracting this condition (Kalia et al., 2016) . Manusia memiliki sistem kekebalan di bawah sel fagositik yang menjadi pertahanan penting terhadap organisme dan sel ganas (Venkatalakshmi & Brindha, 2016). Activation of the immune system needs to be activated in order to play a role in fighting antigenic substances that enter the body. This activation can be stimulated by the administration of immunomodulatory agents.

Immunomodulatory drugs of synthetic origin have shown benefits, but they have also shown adverse side effects so that the use of such drugs has become less effective. Therefore, the development of research to find safer and more effective immunomodulators is very important (Venkatalakshmi & Brindha, 2016). Potential immunomodulatory agents to be developed from plant extracts, where these plant extracts generally have smaller side effects (Alamgir & Uddin, 2010). Immunomodulators derived from plants work by enhancing the capacity and efficacy of natural killer cells, complement, macrophages, granules, and granules, as well as by generating effector molecules (Jayathirtha & Mishra, 2004). Plants have a bladder of metabolite compounds such as flavonoids, alkaloids, sterols, polysaccharides, glycoproteins, and lectins show activity in increasing endurance (Harun et al., 2015).

Plant extracts have been widely studied for their role in various pharmacological effects, including immunomodulatory effects. Quite a potential plant to be developed as a medicinal plant as an immunomodulatory agent is *Cnidoscolus aconitifolius* leaves. Studies have assessed the therapeutic pharmacological properties of *Cnidoscolus aconitifolius* leaves, indicating potential hepatoprotective, antidiabetic, and anticardiovascular effects (Somade et al., 2021). The findings demonstrated that the flavonoid content of CAE with quercetin standard was 418.46±3.28 mgQE/g extract (Hidayati et al., 2023).

T and B cells produce a variety of unique antigen receptors that control the regulated immune system. Through their interaction, surface T cell receptors (TCRs) and MHC molecules enable T lymphocytes to identify certain antigens, transforming them into $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. After activation, $CD4^+$ T cells develop into Th helper cells, which produce Th follicular helper cells (Tfh), T regulatory T cells (Treg), and Th1, Th2, Th9, Th17, and Th22 cytokines. In order to destroy infected cells, the activation of $CD8^+$ cytotoxic T cells can control the production of proinflammatory cytokines and cytotoxic mediators (Poon & Farber, 2020). $CD4^+$ cells, a part of humoral immunity, promote the growth and multiplication of B cells. The function of $CD8^+$ cells within the cell is to cause compatible, infected, or malignant (transplant-rejecting) cells to die. In humoral immunity, $CD4^+$ cells serve to stimulate the growth and multiplication of B lymphocytes. The $CD8^+$ cells work intracellularly by triggering apoptose of infected cells, malignant cells, or compatible histoin cells, which are cells that cause transplant rejection (Baratawidjaja & Rengganis, 2014). As a result, $CD4^+$ and $CD8^+$ are crucial metrics for immune system control and the assessment of immunomodulatory effects.

Flavonoids act as costimulatory agents, effectively reducing the release of chemokines and pro-inflammatory cytokines while concurrently upregulating MHC class II expression (Hosseinzade et al., 2019). In mice fed a Western diet, quercetin treatment can increase plasma leptin and TNF-α, decrease the ratio of $CD4^+/CD8^+$ T cells in the adipose tissue of the epididymis, and limit the increase in macrophages (Kobori et al., 2016). This investigation was carried out in Babl/c mice infected with *Salmonella typhimurium* to ascertain the potential of CAE as an immunomodulator to $CD4^+$ and $CD8^+$ T cells.

MATERIALS AND METHOD

Materials

Salmonella typhimurium bacteria were taken from Brawijaya University, Malang. The ingredients used include Ethanol, $CD4^+$ and $CD8^+$ antibodies, phosphate buffer solutions, and aquadest. Tools used include mouse sonde, surgical instruments, propylene tubes, flowcytometry instruments.

Preparation of *Cnidoscolus aconitifolius* leaves extract

Cnidoscolus aconitifolius Leaves were taken from the Patrang area, Jember. The Biology Laboratory, Faculty of Applied Science and Technology, Ahmad Dahlan University provided the certificate of determination (number 489/Lab. Bio/B/XII/2022). The leaves obtained are wet sorted so that only leaves with a green color are taken, then cleaned and washed using aquadest. After that it is dried in the oven until dry and made powder with a blender. The extraction process begins with sorting the *Cnidoscolus aconitifolius* leaves that have been collected, then the drying process is carried out by aerating for 3 days, then continuing the drying process by ovening at a temperature of 40^{o}C during the formation of dry simplisia and mashed into powder. The finished powder is as much as 500 g, Furthermore, the extraction used 70% ethanol with a total volume of 600 mL for 1 h by ultrasonic maceration, then remaceration is carried out as much as 2 cycle with a volume of 200 mL. The liquid extract obtained is concentrated with a rotary evaporator.

In vivo immunomodulatory activity test in mice

Animal preparation

Thirty male Babl/c strain mice weighing between twenty and thirty grams were housed in the experimental animal laboratory of the Universitas dr. Soebandi Faculty of Health Sciences for a period of fourteen days. This research protocol has been authorized by the Universitas dr. Soebandi Jember's Health Research Ethics Committee under the reference number 096/KEPK/UDS/III/2023. The treatment began by dividing experimental animals into 6 groups, namely the negative control group which were healthy mice without infection, the negative control group which were mice infected with *Salmonella thyparium* bacteria and received placebo CMC Na solution, the positive control group was mice infected with *Salmonella typhimurium* bacteria and received Stimuno® immunomodulator standards, treatment groups 1, 2 and 3 as mice groups infected with *Salmonella typhimurium* bacteria and get CAE doses of 100 mg/kgBW, CAE doses of 200 mg/kgBW and CAE doses of 400 mg/kgBW, respectively. Induction of infection is carried out using *Salmonella typhimurium* bacteria amountingof $1x10^8$ cfu concentration by orally. Three days after induction, mice are examined for bacteremia, including examination of fecal texture and tail blood smear with Giemsa staining. Therapy was started after the mice showed infection. Therapy is administered orally once a day for 7 days (Destiawan et al., 2023).

$CD4^+$ and $CD8^+$ analysis using flow cytometer

$CD4^+$ and $CD8^+$ analysis using the spleen. The spleen organ is washed and crushed in 5 mL of phosphate buffer. After that, it is filtered through the wire and transferred in a 1:3 ratio into a 15 mL propylene tube. The sample was then centrifuged for five minutes at 10°C and 2500 rpm. Take off the supernatant and add 1 mL of phosphate buffer (Kusnul et al., 2017).

After obtaining a 50 µL suspension and centrifuged for 5 minutes at 10°C and 2500 rpm, with the supernatant being disposed of. Incorporate 50 µL of the $CD4^+$ and $CD8^+$ antibody solution, and let it sit at 4°C in the dark for 20 minutes. To prepare a cuvette for flow cytometry analysis, add 400 µL of PBS (Djati et al., 2017).

Analysis of results

Using the SPSS for Windows program, the anova test with significant value ($p<0.05$) was used to analyze the expression of $CD4^+$ and $CD8^+$. Tukey post hoc analysis was used in additional tests to ascertain group differences.

RESULT AND DISCUSSION

The immune response due to *Salmonella typhimurium* bacterial infection is induced by intestinal damage and increased bacterial replication in the intestine. APCs, especially in dendritic cells are the main actors in the immune system's identification mechanism against pathogens. Dendritic cells recognize the lipopolysaccharide structure (LPS) of bacteria via toll-like receptors (TLRs) (Piccioli et al., 2022). Representation of $CD4^+$ T cells via MHC II thereby stimulating increased expression of $CD4^+$ T cells (Leone et al., 2018). However, in *Salmonella typhimurium* bacterial infection, *Salmonella typhimurium* bacteria can inhibit MHC II presentation to $CD4^+$ T cells (Alix et al., 2022) thus, $CD4^+$ T lymphocyte cells are inactive and decrease $CD4^+$ T cell expression when compared to normal (Figure 1 dan Figure 2). This event will lead to excessive systemic infection played by *Salmonella typhimurium* bacteria (Destiawan et al., 2023).

According to the results of flow cytometry analysis, $CD4^+$ T lymphocyte cells express more in lymph tissue cells than $CD8^+$ T lymphocyte cells do. This indicates that $CD4^+$ cells proliferate more quickly than $CD8^+$ cells. This is thought to be because $CD4^+$ plays an important role in secreting various types of cytokines after differentiating into Th1 and Th2. The quantity of $CD4^+$ cells themselves as well as the quantity of other T cells like $CD8^+$ will both increase with a higher $CD4^+$ count (Rachmawati & Rifa'i, 2014) .

In contrast to the negative control group, the results demonstrated that the administration of CAE dosages of 100 mg/kgBW, CAE dosages of 200 mg/kgBW, and CAE dosages of 400 mg/kgBW increased the $CD4^+$ counts but decreased the $CD8^+$ counts (Figure 2). Normal cells shouldn't overexpress the corresponding response since helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) need to experience apoptosis in order to maintain the body's homeostatic parameters (Bolivar-Wagers et al., 2022).

The immune system can quickly control infection, clear pathogens, and inhibit host tissue damage in cases of infection. In preventing such tissue from being damaged and inhibiting the rate and duration of inflammation, cells regulate both innate cells (macrophages, dendritic cells) and the adaptive immune system. When pathogens like viruses invade the body, innate immunity cells will react swiftly by identifying their molecular patterns. This is especially true when viruses are present. In other circumstances, adaptive immune cells play a major role in mediating immune responses that lead to resistance and the development of immunological memory against pathogens. T cells are important components of the adaptive immune response and function as cells that combat infections. Through the expression of adhesion molecules and cytokines, T cells also "help" other cell types. $CD8^+$ mediates the major role of these cells, whereas $CD4^+$ mediates their secondary function (Egawa, 2015).

T cells develop from multipotent progenitors (MPPs) or common lymphoid precursors (CLPs) in the thymus (Pankow & Sun, 2022). T cell differentiation produces subcells namely $CD4^+$ helper cells (Th1 and Th2), cytotoxic CD8+ (CTL/Tc/Ts/Tr/Th3), and memory T cells (Baratawidjaja & Rengganis, 2014).

MPPs or CLPs in the thymus give rise to T cells (Pankow & Sun, 2022). T cell differentiation produces subcells namely $CD4^+$ helper cells (Th1 and Th2), cytotoxic $CD8^+$ (CTL/Tc/Ts/Tr/Th3), and memory T cells (Baratawidjaja & Rengganis, 2014). Because they attach to MHC II and MHC I and activate the TCR, helper T cells and cytotoxic T cells have a significant effect on the immune response. Interactions between dendritic cells and $CD8^+$ cytotoxic T lymphocytes stimulate the latter's proliferation and enhanced synthesis of TNF and IFN-γ, which can kill intracellular antigen-infected cells. Conversely, $CD4^+$ helper T cells control B cells, cytotoxic T cells, and innate immune responses via generating a variety of cytokines (Egawa, 2015).

By encouraging B cells to multiply and develop, $CD4^+$ cells contribute to humoral immunity. As inflammatory mediators, $CD4^+$ Th1 cells secrete TNF, IFN-γ, and IL-2, which aid in the start of the inflammatory process. It has been demonstrated that $CD4^+$ Th2 cells release cytokines such IL-3, IL-4, IL-5, IL-10, and IL-13, which in turn promote and increase the production of T cells and B cell antibodies. Th2 cells are required for activation of B cells by soluble proteins. In addition to Th1 and Th2, other helper T cells have been found, namely Th2, Th9, Th17, Th22, and Follicular Th (Tfh) with diverse roles and functions. Th9 plays a role in the pathophysiology of airway allergies. Th17 secretes

IL-17 which plays a role in neutrophil activation. Th22 plays a role in inflammation in the epidermal cell lining. Tfh is closely related in the regulation of B cell growth. $CD8^+$ cells work intracellularly by triggering apoptosis of infected cells, malignant cells, and compatible histoin cells, which are cells that cause transplant rejection. The $CD8^+$ cells can also directly destroy infected cells under certain conditions (Baratawidjaja & Rengganis, 2014).

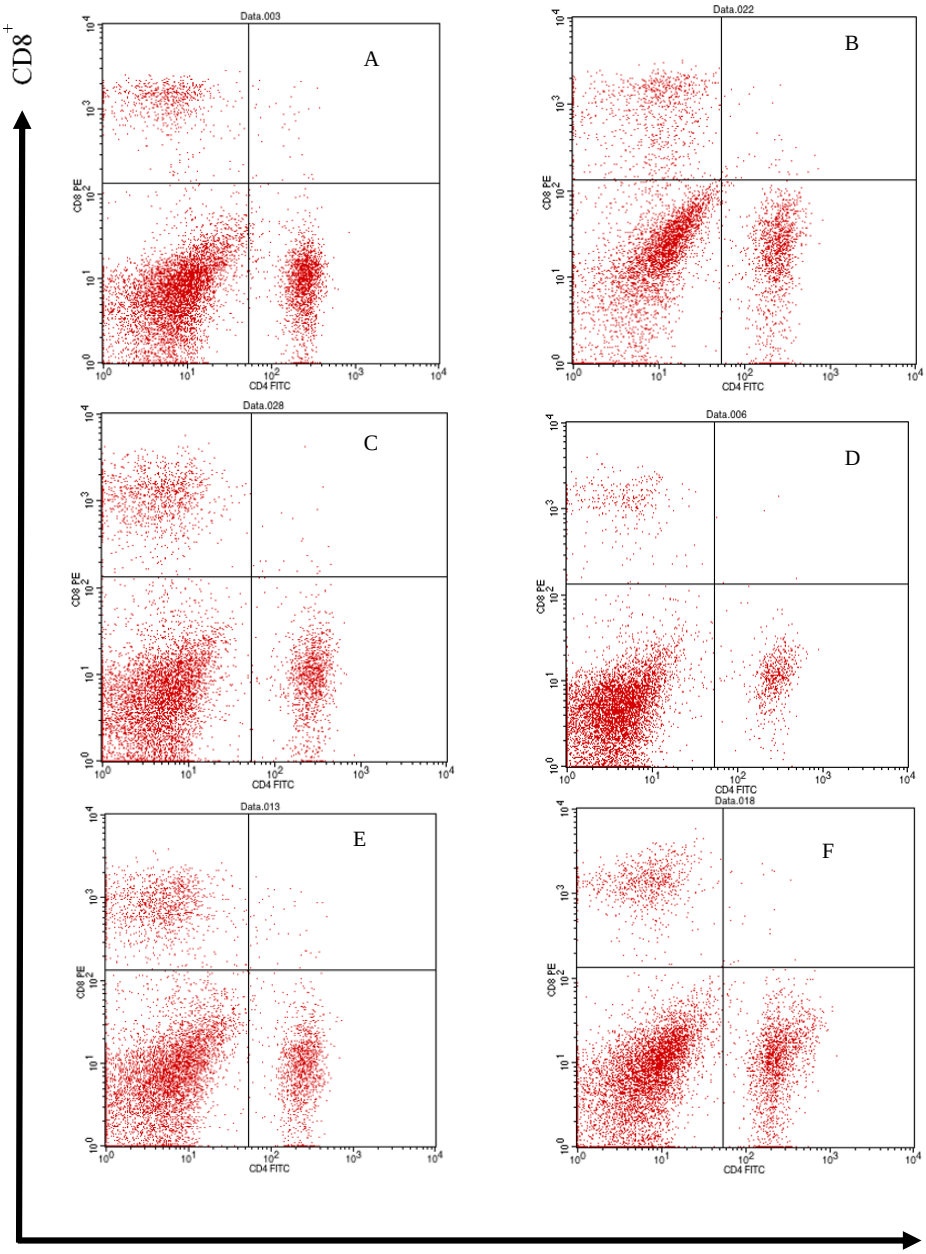

Figure 1. Flow cytometric chart $CD4^+$ dan $CD8^+$ expression post treatment, normal mice (A), negative control (B), positive control (C), CAE dose of 100 mg/kgBW (D), CAE dose of 200 mg/kgBW (E) and CAE dose of 400 mg/kgBW (F)

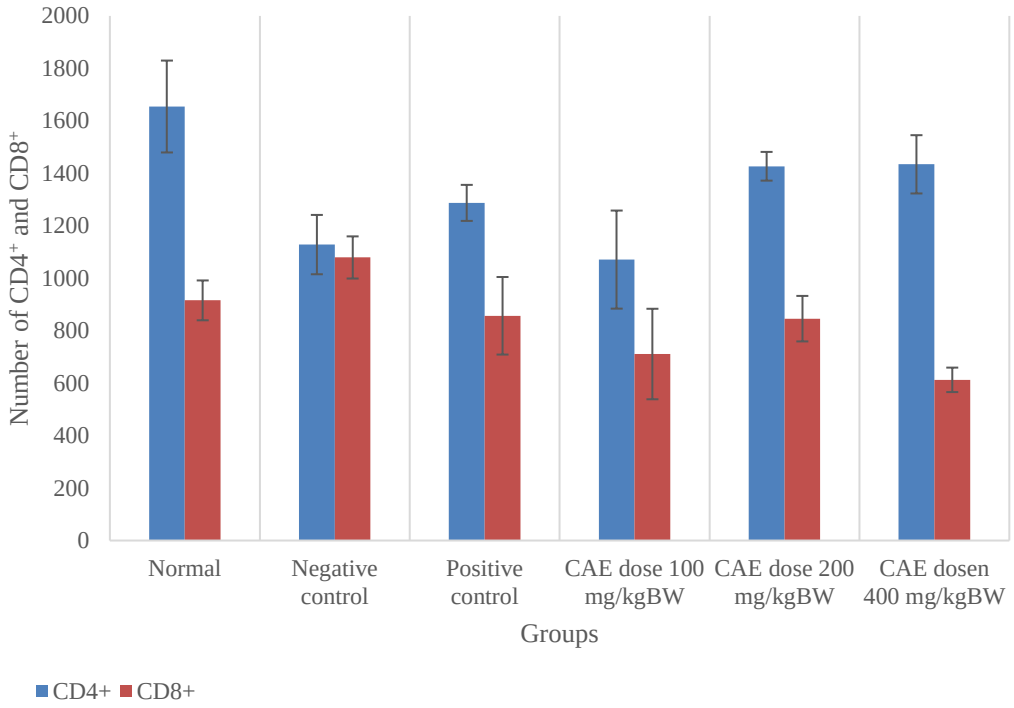

Figure 2. Post-treatment CD4$^+$ and CD8$^+$ expression features analyzed by flow cytometry method in lymph organs

In peripheral blood, the ratio of CD4$^+$/CD8$^+$ in mice and healthy individuals is approximately 2:1; deviations from this ratio may signify immune-related or autoimmune disorders. An immune system malfunction is indicated by a CD4$^+$/CD8$^+$ ratio of less than 1/1, or inverted. Conversely, a greater CD4$^+$/CD8$^+$ ratio is associated with improved immunological activity. A reduction in the ratio suggests a decline in immune system performance as well (Bradshaw et al., 2020). It can be observed from the study results that CAE dosages of 100 mg/kgBW, CAE dosages of 200 mg/kgBW, and CAE dosages of 400 mg/kgBW can raise the expression ratio of CD4$^+$ and CD8$^+$. Notably, as compared to negative controls, the administration doses of 400 mg/kgBW of CAE showed significantly different increases ($p<0.05$) (Table 1). This shows that giving CAE doses of 400 mg/kgBW is very potential to improve immune function.

Table 1. The post-treatment ratio values expression of CD4$^+$ and CD8$^+$ in *Salmonella typhimurium* infected mice

Treatment Groups	Mean CD4$^+$/CD8$^+$ Ratio±SE Values
Normal	1.87±0.30
Negative control	1.10±0.20
Positive control	1.70±0.28
CAE dose 100 mg/kgBW	1.88±0.27
CAE dose 200 mg/kgBW	1.75±0.17
CAE dose 400 mg/kgBW	2.36±0.15*

* Considerably different ($p<0.05$) from the negative control group

Twenty phytochemical substances with a range of medicinal effects are found in CAE, according to its phytochemical study. These substances include the widely distributed 1,2,3-Propanetriol and its

esters, n-Octadecanoic acid, n-Hexadecanoic acid, n-Octacosane, l-(+)-Ascorbic acid-2,6-dihexadecanoate, and 9-Octadecenoic (Z) acid (Omotoso et al., 2024). CAE has high anti-inflammatory activity, can reduce TNF-α expression by 39.78% and IL-6 expression by 97.81% where TNF-α is produced as much as 46% and IL-6 is produced as much as 48.38% in macrophages stimulated by lipopolysaccharides (LPS). This indicates that the bioactive component composition of the extract has the potential to exhibit antioxidant and anti-inflammatory properties in vitro (Us Medina et al., 2019). mTOR, especially in T cells, is a crucial modulator of metabolism and the immune system. Flavonoids have the ability to inhibit mTOR activity, which in turn can trigger a portion of T regulation (Hosseinzade et al., 2019).

CONCLUSION

According to this study, 400 mg/kgBW of CAE may one day be produced as an immunomodulatory drug to improve immune function.

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