Expression of Immunoglobulin M (IgM) and Immunoglobulin G (IgG) in Normal Wistar Rat Post-Cheral® Administration

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1. Introduction

One of the immune system’s main components is immunoglobulin, a class of antibodies that protect the body from pathogenic attacks. The immunoglobulin produced is part of the complex natural defense against pathogens. Immunoglobulin comprises various classes with different roles and functions in maintaining immunity (Megha and Mohanan 2021). The first type of immunoglobulin the body produces is immunoglobulin M (IgM). IgM is created as part of the immune system’s response to bacterial infections in the normal intestine and other tissues. In addition to IgM, there is also immunoglobulin G (IgG), an antibody that can respond to infections and neutralize toxins (Chen et al. 2020). IgM and IgG work in tandem to protect the body from infections and diseases. IgM provides initial protection and rapid response to pathogens, while IgG offers long-term protection and a memory response (Jones et al. 2020). An increase in IgM indicates an initial infection from a pathogen (Hou et al. 2020). Meanwhile, a decrease in IgG can also be due to the influence of toxicants (Ulfman et al. 2018).

One of the efforts to maintain immunoglobulin levels in the body is using herbal medicine to support the body’s immune system. Phyllanthus niruri is commonly called meniran, and this plant has been widely used as an herbal medicine to treat various daily health problems (Hidanah et al. 2022). Traditional people call it a "miracle plant" because all plant parts can potentially treat various diseases. Phyllanthus niruri contains multiple active compounds, with its primary function...
being to boost the body's immune system, mainly through flavonoid compounds. Flavonoids like quercetin, quercitrin, isoquercitrin, astragalin, rutin, kaempferol-4, and rhamnopyranoside can be found in *Phyllanthus* sp. (Fitrotin *et al.* 2021). *Phyllanthus niruri* can increase the population of B and T lymphocytes, macrophages, and lymphocyte activity (Pasaribu *et al.* 2023). *Phyllanthus niruri* has active compounds that can boost the production of certain antibodies and cell proliferation (Harmini *et al.* 2023). Rutin, one of the flavonoid compounds, can exhibit protective effects on humoral immunity by increasing antibody titers or immunoglobulins (Li *et al.* 2023).

*Curcuma longa* is an indigenous herb in Southeast and South Asia. also refers to turmeric. People use it through various preparation forms to treat several diseases (Sharifi-Rad *et al.* 2020). Tumeric’s efficacy as a medicinal drug has been the subject of many scientific studies due to its immunomodulating effects in treating diverse immune-related conditions (Yuandani *et al.* 2021). A previous study reported that *C. longa* improved immune function by reducing levels of pro-inflammatory cytokines and decreasing profile IgE in diabetic rats infected with *Staphylococcus aureus* (Shabana *et al.* 2015). Curcumin is one of the bioactive compounds content in turmeric and can also indirectly increase the expression of immunoglobulins, including IgM and IgG. Recent research in autoimmune models shows that curcumin can modulate T helper cells (Allegra *et al.* 2022). The mechanism is that T helper cells will help the proliferation of B cells by increasing the production of plasma cells that can express immunoglobulins (Cyster and Allen 2019).

Cheral® is an herbal product manufactured by PT Ismut Fitomedika Indonesia. It contains two herbs, *Phyllanthus niruri* and *Curcuma longa*. Compounds within these two herbs have shown potential as immunomodulators (Puspitarini *et al.* 2019) by maintaining the levels of immunoglobulin M and G in the body. Therefore, it is essential to understand the effects of Cheral® administration on immunoglobulin levels in normal animal models. Consequently, this research aims to determine the levels of immunoglobulin M and G in healthy rats after the administration of Cheral®.

2. Materials and Methods

2.1. Experimental Design

This research employed a completely randomized design, encompassing experimental research. The research was conducted in vivo using four treatment groups, with six rats in each group. All groups of experimental animals in this study were healthy and normal. The four groups included the control group (administered distilled water orally), dose 1 (Cheral® administered orally at 156.25 mg/Kg BW), dose 2 (Cheral® administered orally at 312.5 mg/Kg BW), and dose 3 (Cheral® administered orally at 468.75 mg/Kg BW). The dosage used in this study was determined following the instructions on the Cheral® product (500 mg three times a day). This dosage was then converted from human to animal based on FDA standards. The converted dosage is 156.25 mg/kg BW, used as dose 1. Subsequently, the determination of doses 2 and 3 resulted from modifying dose 1. Dose 2 is the outcome of doubling dose 1, while dose 3 is tripling dose 1. All groups were treated for 90 days. Subsequently, the rats were sacrificed, and spleen organs were collected to observe the effects on the immunoglobulins (IgM and IgG) expressed by B cells.

2.2. Animal

This research utilized male Wistar rats (*Rattus norvegicus*) sourced from Murine Farm, Singosari, Malang, Indonesia. The rats selected for the study were between 6 and 8 weeks old and had a minimum body weight of 120 grams. These experimental animals were healthy, characterized by their activity, alertness, clear eyes, straight and undamaged limbs, dense, white, and smooth fur, and a normal gait. The rats were housed in plastic tubs serving as cages, equipped with wire covers and bedding made of husk (wood shavings), which was replaced every 2-3 days. Cage conditions were maintained to minimize contamination as described by Adharini *et al.* (2020). The rats were provided with an adequate supply of food and water.

2.3. Oral Administration of Cheral®

This research received evaluation and approval from the Ethics Commission of Brawijaya University, Malang, Indonesia, with reference number 021-KEP-
UB-2023. Cheral® is an herbal product in powder form, obtained from PT. Ismut Fitomedika Indonesia (IFI), Makassar, Indonesia. Cheral® is a product containing a combination of Curcuma longa and Phyllantus niruri, with a dosage ratio of 50:50. The oral administration of Cheral® was conducted over a total period of 90 days with 5 times oral administration a week. Cheral® was dissolved in distilled water at a volume of 2 ml and administered to rats with a body weight of 200 grams.

2.4. Spleen Organ Isolation, Immunostaining, and Flow Cytometry
All treated rats were sacrificed on day 91, and the B cells expressing immunoglobulins were isolated from spleen organs. The spleen organs were rinsed with Phosphate Buffered Saline (PBS) and placed in a petri dish containing 3 ml of PBS, where they were subsequently crushed. The rinsed spleen was carefully collected without organ debris and transferred to a 15 ml polypropylene tube. The tube was then centrifuged at 2,500 rpm and a temperature of 10°C for 5 minutes. The resulting pellet was resuspended with 1 ml of PBS. A 50 μL aliquot of the resuspension was divided into microtubes for immunostaining, followed by flow cytometry analysis. The immunostaining and flow cytometry procedures carried out in this study followed the procedures carried out by Adharini et al. (2020).

2.5. Data Analysis
The data obtained from flow cytometry was subjected to statistical analysis using the SPSS program. The analysis involved a one-way analysis of variance (ANOVA) and was subsequently followed by a post hoc test to determine statistical significance. The significance level for the post hoc test was set at a p-value less than 0.05, as per the methodology described by Adharini et al. (2020).

3. Results
This study involved a combination of staining with CD19 antibodies to determine the IgM and IgG profiles. Flow cytometry analysis enables the identification and quantification of B cell subpopulations in the sample by using specific anti-CD19 antibodies that bind to the surface proteins of B cells. Based on statistical analysis shows that the immunoglobulin levels are homogeneous, and the p-value is more than 0.05. It is shown that there were no spike values between the treatment of individual groups and that of the control group, indicating that there were no infections during the experiment. The physical health of each rat before being sacrificed showed no defects, bright eyes, active movement, normal behavior, white fur, smooth and not falling out, and normal feces, which are indicators of a healthy rat. IgM is one of the B cells' products. The relative number of IgM cells in the Cheral® D2 (38.71%) and D3 (36.08%) treatment groups did not show a significant difference compared to the control treatment group (44.40%). However, dose 1, with a relatively lower amount (24.18%), exhibited a significant difference compared to the control and D2 treatment groups (Figure 1). It's important to note that these results show that the D1 treatment group has a lower relative number of IgM cells and is significantly different from the control and D2 treatment groups. This suggests that the different Cheral® doses may affect the IgM production by B cells. In addition, the IgM value of the treatment was lower than the control, indicating that Cheral® was able to maintain the health condition of rats in fighting pathogens.

The relative number of IgG cells did not exhibit significant differences between the Cheral® treatment and control groups (Figure 2). In comparison, the relative number of IgG cells in group D3 was lower than in groups D1 and D2. The control group had a value of 44.26%, D1 had 46.87%, D2 had 49.34%, and D3 had 29.21%. The comparison between the treatment and control groups, indicating a higher IgG profile in the treatment group, suggests that Cheral® may have contributed to enhancing the body’s immune system. This improvement could be attributed to Cheral® potential role in supporting the formation of antibodies, thereby aiding in the fight against pathogenic threats.
Figure 1. The relative number of CD19+IgM+ (IgM) after Cheral administration on 90 days. (A) Spleen cells (2 ×10^6 ) were obtained from all rats, then subjected to extracellular staining cells with anti-CD19 antibody and intracellular staining cells with anti-IgM antibody and analyzed by flow cytometry. N: control (administered distilled water orally), D1: Dose 1 (Cheral® administered orally at 156.25 mg/Kg BW), D2: dose 2 (Cheral® administered orally at 312.5 mg/Kg BW), and D3: dose 3 (Cheral® administered orally at 468.75 mg/Kg BW), (B) the graphic bar is a calculation of CD19+IgM+ in splenic cells. The data are mean value ± SD of six rats in each group with a significant value <0.05 (n = 24)

Figure 2. The relative number of CD19+IgG+ (IgG) after Cheral administration on 90 days. (A) Spleen cells (2 ×10^6 ) were obtained from all rats, then subjected to extracellular staining cells with anti-CD19 antibody and intracellular staining cells with anti-IgG antibody and analyzed by flow cytometry. N: control (administered distilled water orally), D1: Dose 1 (Cheral® administered orally at 156.25 mg/Kg BW), D2: dose 2 (Cheral® administered orally at 312.5 mg/Kg BW), and D3: dose 3 (Cheral® administered orally at 468.75 mg/Kg BW), (B) the graphic bar is a calculation of CD19+IgG+ in splenic cells. The data are mean value ± SD of six rats in each group with a significant value <0.05 (n = 24)
4. Discussion

The analysis of IgM and IgG expression after the administration of Cheral® was conducted over a 90-day treatment period to investigate the long-term effects of daily Cheral® consumption on IgG and IgM profiles. Cheral® claims to function as an immunomodulator. The IgG and IgM profiles show that the administration of Cheral® can maintain immunoglobulin levels in healthy rats during treatment. IgM is expressed as early as B cell development. IgM has a lower affinity due to its role as a natural antibody. Despite this, IgM is crucial in the initial defense line and immunoregulation (Liu et al. 2019). The IgM profile in this study shows that Cheral® is assumed to be able to maintain the health condition of rats as a primary immune response, as indicated by the low IgM profile. High IgM levels indicate an early-phase infection (Aljabr et al. 2022). IgM production is carried out to prevent the movement of pathogens in the body and facilitate phagocytosis (Perez et al. 2023). In addition, IgM is a strong aglinator against antigens and the presence of IgM is very important for the body.

IgG antibodies directly contribute to the immune response, involving the neutralization of toxins and viruses. IgG plays a pivotal role in combating pathogens, exhibiting a higher affinity than IgM (Napodano et al. 2021). The IgG profile in this study shows that dose 1 and dose 2 have a high profile compared to the control. This shows that these doses can encourage the formation of antibodies in the body that play an important role in fighting infected pathogens. Whereas dose 3 of the compound in Cheral® is toxic in the long run, which can reduce the IgG profile. In addition, a spike in IgG count that is too high may also be associated with an autoimmune response (Thompson et al. 2023).

Based on research results, Cheral® has a potential to maintain the immune system. Cheral® is known to be produced from Phyllanthus niruri and Curcuma longa. Both herbs generally contain various flavonoid compounds (Puspitarini et al. 2019). Flavonoids can activate various signaling, including the Mitogen Activated Protein Kinase (MAPK) pathway signaling. The best pathway that plays a role in cell proliferation and differentiation is Rapidly accelerated fibrosarcoma -Mitogen-activated protein kinase –Extracellular signal-regulated kinase (Raf-MEK-ERK). (Zulkefi et al. 2023). Flavonoids can also increase Major Histocompatibility Complex Class II (MHC II) expression on the surface of B cells (Pagano et al. 2022), where T helper cells will recognize MHC II and assist in B cell proliferation and interleukin (IL) production, which can ultimately increase the production of effector B cells (plasma cells) that can secrete antibodies (Schultheiß et al. 2022). Curcumin contained in turmeric also plays a role in B cell proliferation (Badr et al. 2020). Flavonoid compounds can exert dual regulatory effects under different conditions on B cells. Flavonoids can play a role in stimulating the expression of immune cells and suppressors, thus maintaining the immune system under normal conditions (Han et al. 2021).

Several factors can cause low relative amount of IgM cells. In normal people, IgM is sometimes found with lower levels than in general. Another factor is due to viral and bacterial infections that cause low IgM levels (Wang et al. 2023). Similar mechanisms may also apply in healthy rats. In addition, low doses of some drugs or compounds in individuals with stable or healthy health, work effectively, tolerably, minimize side effects and long-term use. Immunoglobulins are essential to humoral immunity and play a significant role in the immune system's mechanisms. Immunoglobulin levels are frequently used to represent the humoral response status in clinical practice (Chen et al. 2020). Therefore, it is crucial to continuously maintain the levels of IgM and IgG in the body.

In conclusion, administration of Cheral® in various doses did not significantly affect the relative number of IgM and IgG cells compared to controls, indicating that Cheral® administration does not cause toxic effects and maintains homeostasis of immunoglobulin expression. Furthermore, doses 1 and 2 showed a lower IgM profile than the control and higher IgG when compared to the control, indicating the formation of antibodies against the pathogen. However, further research on Cheral® as an immunomodulator needs to be done in more depth by looking at the overall profile of immune cells.

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