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Anti-inflammatory Activity of Glucomannan Resulting from β-mannanase Enzyme Hydrolysis in RAW 264.7 Cells

Nuraini Nuraini[1,2*], Effionora Anwar [1], Dian Ratih Laksmitawati[1], Hendig Winarno[1,3], Siti Irma Rahmawati[4]

[1] *Faculty of Pharmacy, Universitas Pancasila, South Jakarta, DKI Jakarta, Indonesia*
[2] *Faculty of Pharmacy, Universitas Muhammadiyah A.R. Fachruddin, Tangerang, Banten, Indonesia*
[3] *Research Center for Radiation Process Technology, Research Organization for Nuclear Energy, National Research and Innovation Agency, KST BJ Habibie, South Tangerang, Banten, Indonesia*
[4] *Research Center for Vaccine and Drugs, Research Organization for Health, National Research and Innovation Agency (BRIN), Jalan Raya Jakarta-Bogor Km. 46, 16911 West Java, Indonesia*
*Corresponding Author: nuraini.apt@unimar.ac.id

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ABSTRACT: Glucomannan is a polysaccharide of the hemicellulose type, composed of glucose and mannose chains. It has the ability to form solutions that expand and thicken. The bioactivity of carbohydrates is influenced by the length of their saccharide chains. Hydrolysis can alter the structural dimensions of polysaccharides, including molecular weight, type, number, and position of substituent groups, which in turn impacts their biological activity. Various methods can modify carbohydrate structures to enhance their value, with degradation being one such method. Enzymatic degradation is particularly advantageous due to its high specificity, efficiency, and minimal side effects. Numerous studies have identified glucomannan as having immunomodulatory effects on macrophage cells. When stimulated with lipopolysaccharides, macrophages release various cytokines and inflammatory mediators, such as TNF-α, iNOS, and NO. This study explored the potential anti-inflammatory effects of glucomannan (GM) and its enzymatically hydrolyzed form (Enz-GM) on RAW 264.7 cells. The research involved degrading glucomannan using the β-mannanase enzyme, followed by in vitro testing of the degradation product (Enz-GM) for anti-inflammatory activity. This was achieved using an ELISA kit to measure the expression of IL-6, TNF-α, and iNOS. The results showed that Enz-GM at pH 9 (Mw 1.24 kDa) significantly reduced the pro-inflammatory cytokines IL-6 and iNOS ($p \leq 0.05$) compared to GM, while TNF-α expression did not show a significant difference between Enz-GM and GM.

KEYWORDS: Antiinflammatory; Cytokines; Glucomannan; Hydrolysis; Macrophages.

1. INTRODUCTION

Carbohydrates have been recognized as an essential source of food for a long time, and they are also commonly utilized in the health sector. Various carbohydrates and their hydrolysis products are currently employed as immunomodulators, including glucan, trehalose dimycolate (TDM), muramyl dipeptide (MDP), and lipopolysaccharide [1]. Tubers are a common source of carbohydrates, and porang tubers are particularly abundant in Indonesia. These tubers are derived from the porang plant (*Amorphophallus muelleri Blume*), which contains the carbohydrate glucomannan. Glucomannan is a hemicellulose-type polysaccharide made up of chains of glucose and mannose [2].

Glucomannan offers numerous health benefits, such as acting as a natural antioxidant, providing a source of carbohydrates with a low glycemic index, serving as a prebiotic, and functioning as an immunomodulator. It also has anti-fatigue, antiviral, anti-inflammatory, and anti-obesity properties, helps lower LDL cholesterol, and aids in the healing of diabetic wounds [3-12]. Both in vivo and in vitro research have been conducted, with many in vitro studies focusing on macrophage cells. Macrophages are part of the innate immune system and play a crucial role in both innate and adaptive immune regulation. They are involved in various functions, including phagocytosis, antigen presentation, and the production of different cytokines. IL-6 is a critical

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signaling molecule that regulates the acute phase response, promoting T cell expansion and activity, as well as B cell differentiation. TNF-α is one of the most significant cytokines involved in regulating acute local and systemic immune and inflammatory responses. In summary, IL-6 and TNF-α are proinflammatory cytokines essential for immune system regulation [13]. Enzymatic degradation offers advantages over chemical degradation, including high specificity, efficiency, and minimal side effects. After enzymatic degradation, the polysaccharide product typically has a uniform relative molecular mass, which does not significantly impact the main chain of the polysaccharide.

The primary process of enzymatic degradation involves breaking down the polysaccharide backbone, thereby reducing its molecular weight and viscosity. Currently, enzymatic modification is mostly limited to the degradation of specific types of polysaccharides. The development of other enzyme types, such as transferases and synthases, could expand the applications of enzyme technology for polysaccharide structural modifications [14]. This study investigated the potential anti-inflammatory effects of glucomannan (GM) and its hydrolysis product (Enz-GM) as part of an immune response, using RAW 264.7 cells as the test model. RAW 264.7 cells are macrophage cells derived from male mice that have been induced with Abelson murine leukemia virus tumors.

2. MATERIALS AND METHODS

2.1. Materials

Glucomannan powder was obtained from PT. Paidi Indo Porang, East Java - Indonesia, aqua destilata (Bratachem, Indonesia), natrium carbonat (Bratachem, Indonesia), β-mannanase enzyme (Xian Henrikang Biotech, China), phosphate-buffered saline/PBS (Biowest, USA), DMSO (Merck, German), Thiazolyl blue tetrazolium bromide (Sigma Aldrich, USA), methanol (Merck, German), MTT reagen (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) (Sigma Aldrich, USA), bradford reagen (Sigma Aldrich, USA), griess reagent (Sigma Aldrich, USA), biotynilated detection ab (Elabscience, USA), HRP conjugate (Elabscience, USA), wash buffer solution (Elabscience, USA), substrate reagent (Elabscience, USA), stop solution (Elabscience, USA), standard solution (Elabscience, USA), sulphate Solution (Elabscience, USA), alkali reagent (Elabscience, USA), chromogenic agent A (Elabscience, USA), chromogenic agent B (Elabscience, USA), RAW 264.7 cell line (ECACC 91062702, UK), , dulbecco's modified Eagle's medium High Glucose/DMEM High Glucose (Gibco, USA), fetal bovine serum Premium/FBS (Biowest, USA), antibiotic dan antimycotic/ABAM (Sigma Aldrich, USA).

2.2. Procedure

2.2.1. Glucomannan hydrolysis using the β-mannanase Enzyme

The enzymatic hydrolysis method for glucomannan uses the enzyme β-mannanase with modification of temperature and incubation time and pH variations [15][16].
A total of 20 g of glucomannan powder was dissolved in 1 L of distilled water, resulting in a solution with a pH of 5.28. Then, 20 g of β-mannanase enzyme, which had a pH of 5.69, was added and mixed thoroughly to initiate the reaction. The mixture was adjusted to pH 5.5 and incubated at 55°C for 5 hours. To terminate the degradation process, the reaction mixture was boiled for 15 minutes and then centrifuged at 4000 rpm. The sample was then frozen in a freezer and freeze-dried until a dry product was obtained. For hydrolysis under neutral and alkaline conditions, 20 mL of Na_2CO_3 was added to adjust the pH to 9, followed by incubation and processing using the same procedure as for the acidic conditions.

2.2.2. Molecular weight determination using Gel Permeation Chromatography

The first step involves preparing the gel by developing it in an appropriate solvent. The gel used in Gel Permeation Chromatography (GPC) is a semi-permeable polymer with a defined range of pore sizes. Once the gel expands, it is packed into columns. Gel beads with a specified pore size range are carefully arranged in the column to create a porous matrix for separation. After packing, it is crucial to remove any air bubbles and check the uniformity of the column. Several column volumes of buffer solution are passed through to wash and stabilize the column. Once prepared, the next step is to load the glucomannan sample onto the column. The sample is injected at the top of the column using a syringe and allowed to flow into the gel matrix.

Following sample loading, the elution process starts. During elution, components of the sample are detected by appropriate detectors. These detectors measure specific properties of the eluted components, providing data on their concentration, molecular weight, or other relevant characteristics. The resulting chromatogram displays the distribution of the polymer or analyte's weight in relation to retention volume.

2.2.3. Culturing of RAW 264.7 cells

Cells were retrieved from vials stored at -80°C and then thawed in a water bath at 37°C for 2 minutes until fully liquid. The cells were transferred to a 15 mL centrifuge tube containing 4 mL of culture medium. The RAW 264.7 cell culture medium was prepared by mixing DMEM high-glucose basal medium with 10% FBS and 1% antibiotics and antimycotics. The cells were centrifuged at 1500 rpm for 5 minutes, after which the supernatant was discarded, and the pellet was resuspended in 4 mL of culture medium. The cell suspension was then placed into a T25 flask and incubated in a 5% CO_2 incubator at 37°C. The cells were monitored under an inverted microscope until they reached approximately 70-80% confluence in the T25 flask. The culture medium was removed, and the cells were washed with PBS. Adherent cells were detached using a scraper and checked under an inverted microscope to ensure complete detachment from the flask surface. The cell suspension was transferred to a 15 mL centrifuge tube, which was then centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 1 mL of culture medium. Cell density was calculated by mixing 100 µL of the cell suspension with 100 µL of trypan blue dye (1:1) in a 1.5 mL tube and resuspending it thoroughly. Next, 10 µL of the cell mixture was placed into a hemocytometer, which was examined under an inverted microscope to count the number of viable cells. Trypan blue stains only dead cells, making them appear blue, while live cells remain unstained. The cell density obtained (number of living cells) determined the quantity to be seeded into a flask. A cell density of approximately 1 x 106 cells/mL was used for one T25 flask. If the cell density was ≤ 2 x 106 cells/mL, the cell suspension was transferred to a T75 flask. Once transferred to the appropriate flask, cells were incubated in a 5% CO_2 incubator at 37°C. During treatment, the growth medium was replaced or replenished every 2-3 days.

2.2.4. Anti-inflammatory activity on RAW 264.7 cell

The in vitro anti-inflammatory activity was conducted at the Invilab laboratory in Bogor, West Java, Indonesia. RAW 264.7 cells were counted using a hemocytometer and seeded at a density of 5 x 105 cells per well in 6-well plates. The cells were then incubated for 24 hours at 37°C with 5% CO_2. After 24 hours, the culture medium was replaced with 1600 µL of fresh culture medium, and 200 µL of the extract sample was added to each well, followed by incubation for 1-2 hours at 37°C with 5% CO_2. Subsequently, 200 µL of LPS (1 µg/mL) was added to each well, and the cells were incubated for another 24 hours at 37°C with 5% CO_2. After the incubation period, the conditioned medium (CM) was collected into 1.5 mL tubes and stored at -80°C for future analysis. Prior to testing, the CM was centrifuged at 2000 x g for 10 minutes to separate the supernatant from the cells. Determination of parameters such as NO, TNF-α, and IL-6 was performed with modifications to accommodate differences between ELISA Kit manufacturers [4].

The standard solution was added to each well twice, 100 µL per addition. The wells were sealed and incubated for 90 minutes at 37°C. After incubation, the solution was removed, and 100 µL of Biotinylated Detection Antibody solution was added to each well. The wells were covered and incubated for 1 hour at 37°C. The solution was discarded, and the wells were washed with wash buffer; the buffer was left for 1-2 minutes before being discarded. This washing step was repeated three times. After washing, 100 µL of HRP conjugate was added to each well, the plate was covered, and incubation was carried out for 30 minutes at 37°C. The plate was washed five times, and then 90 µL of substrate solution was added to each well and incubated for 15 minutes at 37°C. The reaction was monitored for color change; if it did not occur, incubation was extended, but not for more than 30 minutes. To stop the reaction, 50 µL of stop solution was added to each well. The optical density (OD) was measured at 450 nm using a microplate reader (Tecan, Swiss). Absorbance values were recorded at 450 nm, and cytokine concentrations were determined by comparing the results with a standard curve.

2.2.5. Data analysis

Data analysis was performed using SPSS with a one-way ANOVA followed by Tukey's post hoc test to assess the differences between enzymatically hydrolyzed glucomannan (Enz-GM), glucomannan, and LPS. Results were considered significant if the p-value was ≤ 0.05.

3. RESULTS

3.1. The result of glucomannan hydrolysis using the β-mannanase enzyme

The outcomes of glucomannan hydrolysis were achieved by hydrolyzing glucomannan (Enz-GM) at pH 5.5, pH 7, and pH 9, with an incubation temperature of 55°C for 5 hours (table 1).

3.2. Molecular weight of Gel Permeation Chromatography (GPC) results

According to the principle of GPC, larger molecules have more restricted access to the stationary phase in the chromatography column (permeable gel), causing them to move through the column more quickly and elute earlier than smaller molecules. In contrast, smaller molecules can enter the gel pores and take longer to traverse the column. This mechanism enables the separation of molecules based on size, allowing for the observation of differences in peak appearances between crude glucomannan (GM) and hydrolyzed glucomannan (Enz-GM), depending on the molecule size in this study.

Table 1. Molecular weight of glucomannan and glucomannan resulting from enzyme hydrolysis.

Sample	Variation	Molecular weight (kDa) and curve area (%)							
		component 1 (≥Mw 100001)	**component 2 (Mw 10001 – Mw 100000)**	**component 3 (Mw 1001 – Mw 10000)**	**component 4 (Mw 101 – Mw 1000)**	**component 5 (Mw 29 – Mw 100)**	**component 6 (Mw 5.01 – Mw 29)**	**component 7 (Mw 0.6 – Mw 5.00)**	**component 8 (Mw ≤0.5)**
GM	-	-	-	44087 (14.42%)	**125 (85.58%)**	-	-	-	-
Enz-GM	pH 5.5	257755 (3.10%)	-	-	-	45 (6.94%)	-	**0.79 (89.96%)**	-
	pH 7	236927 (0.96%)	-	-	-	59 (14.54%)	11 (1.44%)	**1.09 (75.90%)**	0.02 (7.16%)
	pH 9	162329 (1.60%)	-	-	-	48 (11.30%)	15 (4.79%)	**1.24 (77.56%)**	0.02 (4.75%)

The comparison of molecular weight (Mw) between crude glucomannan and enzymatically hydrolyzed glucomannan (Enz-GM) can be observed in the different peaks representing various component groups. In the Enz-GM sample, a peak was detected in the first component group with an Mw range of 162,329 kDa to 257,755 kDa, which was significantly higher than the Mw of crude glucomannan (GM), initially identified in the third component group at an Mw of 44,087 kDa. However, the Mw of Enz-GM gradually decreased from the fifth to the eighth component group. This decrease in Mw varied and was influenced by the pH conditions during the enzymatic hydrolysis. The Mw of Enz-GM at pH 5.5 was 0.79 kDa, at pH 7 it was 1.09 kDa, and at pH 9 it was 1.24 kDa, with these particles appearing in component 7, which had the largest area (Table 1).

3.3. RAW 264.7 cell culture's results

Cell culture is the process of maintaining living cells in a controlled medium to allow their development and growth in vitro. This growth requires a medium that provides essential nutrients to the cells [17]. In this study, RAW 264.7 cells were used, which are macrophage cells derived from Abelson virus leukemia-transformed cells in BALB/c mice. These cells have the ability to perform phagocytic pinocytosis [18]. Figure 1 displays RAW 264.7 cells after subculturing, which were 80% confluent and ready for testing.

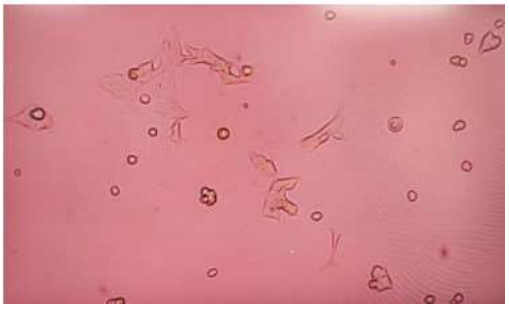

Figure 1. RAW 264.7 cells after culture

3.4. Anti-inflammatory activity on RAW 264.7 cell

The IL-6 ELISA test results for Enz-GM indicated an increase compared to LPS. The average IL-6 concentration for GM was 6769.33 pg/mL, while for Enz-GM, it ranged from 5317.67 pg/mL to 5630 pg/mL. The variation in IL-6 concentration levels is illustrated in Figure 2.

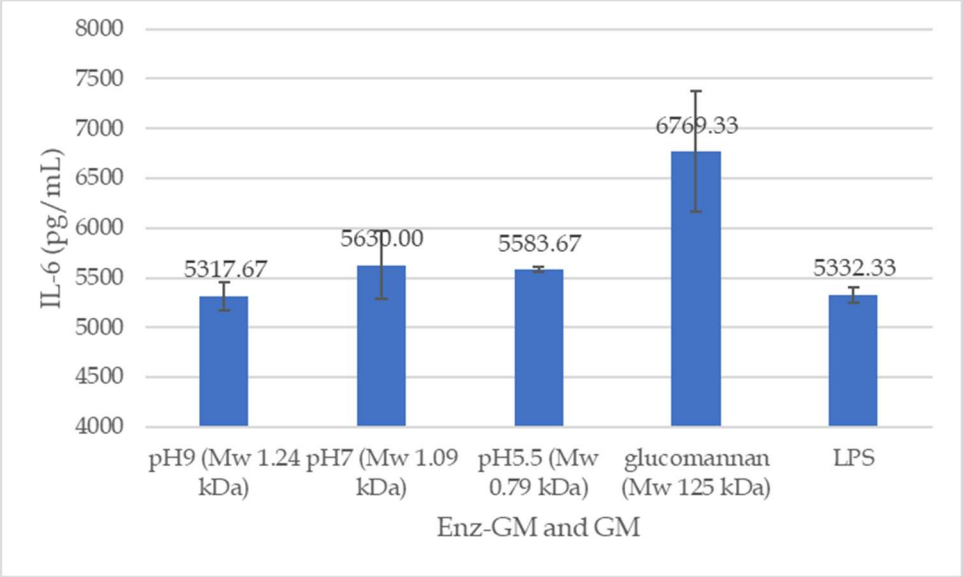

Figure 2. Effect of Enz-GM and GM on IL-6 expression.

The TNF-α expression results showed variations in the production levels across different test samples of Enz-GM, indicating both increases and decreases in TNF-α production. The impact of Enz-GM on TNF-α production varied, with a noticeable decrease in TNF-α levels in GM compared to the positive control (LPS). The average TNF-α concentration for glucomannan was 3726.33 pg/mL, which was higher than the average TNF-α concentration produced by Enz-GM, ranging from 3557 pg/mL to 3797 pg/mL. The differences in these results are depicted in Figure 3.

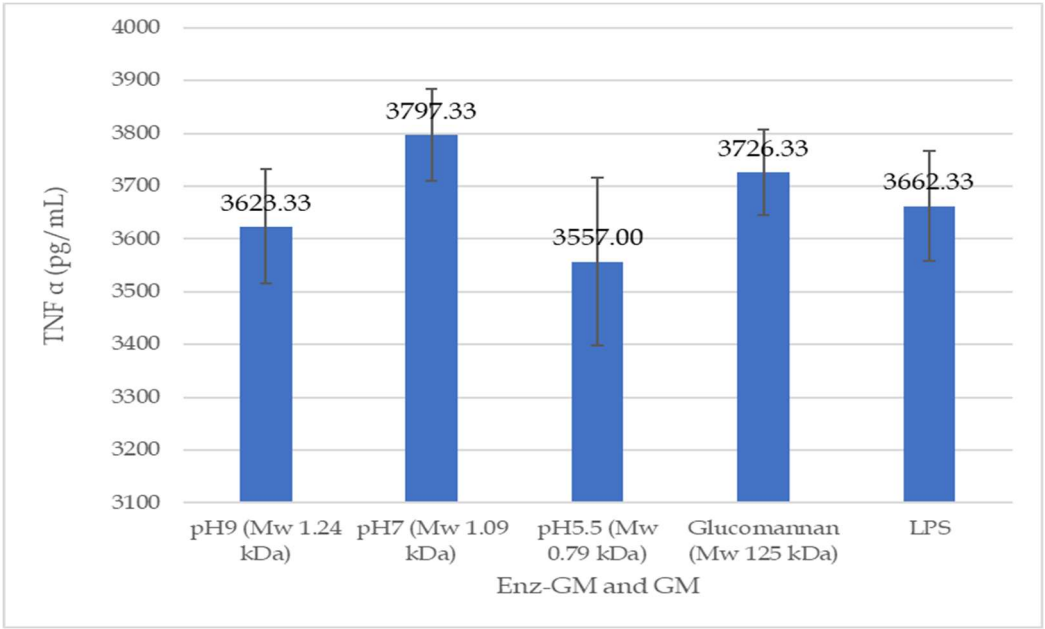

Figure 3. Effect of Enz-GM and GM on TNF-α expression.

The iNOS test is conducted to assess its role in combating infections and regulating the immune system's response to pathogens. The research data indicated a reduction in iNOS concentration in Enz-GM. The

average iNOS expression for GM was 0.0606 ng/mL, whereas for Enz-GM, it ranged from 0.0336 ng/mL to 0.0617 ng/mL. The variations in iNOS levels are illustrated in Figure 4.

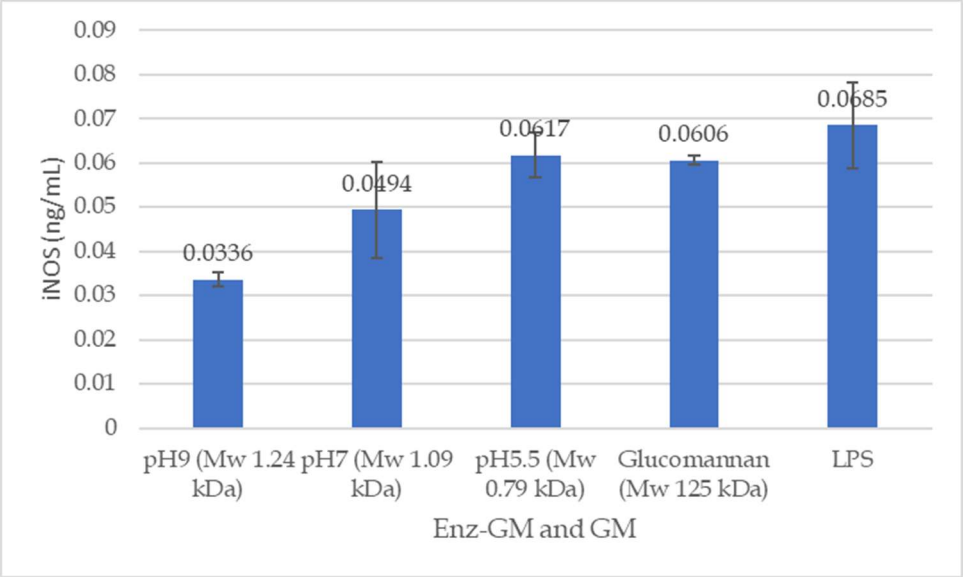

Figure 4. Effect of Enz-GM and GM on iNOS expression.

3.5. Data analysis

The statistical analysis of the Enz-GM group indicated significant differences in comparison to GM across all pH treatments, with significance values of pH 9 ($p = 0.002$), pH 7 ($p = 0.009$), and pH 5.5 ($p = 0.007$). However, when Enz-GM was compared to LPS, there were no significant differences ($p \geq 0.005$). When examining TNF-α expression, no significant differences were found between Enz-GM and GM, with significance values of pH 9 ($p = 0.785$), pH 7 ($p = 0.930$), and pH 5.5 ($p = 0.390$). Similarly, there was no significant difference between Enz-GM and LPS, with a significance value of $p \geq 0.005$. The Tukey post hoc test showed a significant difference in the Enz-GM group compared to GM specifically at pH 9 ($p = 0.005$). Additionally, Enz-GM at pH 9 ($p = 0.001$) and pH 7 ($p = 0.046$) displayed significant differences when compared to LPS.

4. DISCUSSION

Enzymatic degradation at pH 5.5 resulted in the lowest molecular weight (mw) of 0.79 kDa, achieved at an incubation temperature of 55°C with the largest area observed. Notably, Mw values showed minimal variation across pH level of 5.5, 7 and 9. This aligns with research by Chen et al., which highlights that the β-mannanase enzyme exhibits a broad temperature activity range from 30°C to 70°C. The optimal temp for hydrolyzing glucomannan into oligoglucomannan is 40°C, with the highest production of reducing sugars occuring between 40°C and 60°C [15]. Similarly, Anggela et al. reported that β-mannanase enzyme activity and stability are optimal at acidic levels, particularly between pH 5 and pH 6. For porang hydrolysis, the ideal pH is 5.5 [19].

Macrophages play a crucial role in the defense against a variety of microorganisms, including bacteria, viruses, and fungi. When stimulated with lipopolysaccharide, macrophages release a range of cytokines and inflammatory mediators, such as TNF-α, iNOS, and NO [20]. In RAW 264.7 cell culture, the media serves as a growth medium and provides essential nutrients for the cells. The media consists of DMEM, FBS, and antibiotics with antimycotics. DMEM is used to supply nutrients as it contains four times the amount of vitamins and amino acids compared to other media. FBS acts as a serum to enhance growth factors, while the addition of antibiotics and antimycotics helps minimize bacterial and fungal contamination [21].

Macrophage cells are integral components of the innate immune system and act as specialized phagocytes. They generate reactive oxygen species (ROS), which can lead to the production of interleukin-1β (IL-1β), TNF-α, and IL-6. One type of ROS is nitric oxide (NO), known for its antimicrobial properties. NO is synthesized by inducible nitric oxide synthase (iNOS), which is activated in response to microbial, toxin, or antigen stimulation. Additionally, NO is produced by a constitutive gene that is typically expressed under

normal conditions. However, during excessive antigen stimulation, iNOS can also be upregulated and expressed [22]. Considering the characteristics of macrophage cells, this study selected IL-6, TNF-α, and iNOS as the parameters to assess anti-inflammatory activity.

IL-6 production was reduced in Enz-GM compared to GM. The lowest average IL-6 expression was observed in Enz-GM pH 9 (Mw 1.24 kDa) with an average of 5317.67 pg/mL, followed by Enz-GM pH 5.5 (Mw 0.79 kDa) with an average of 5583.67 pg/mL, and Enz-GM pH 7 (Mw 1.09 kDa) with an average of 5630 pg/mL. These levels were lower than the IL-6 expression seen in GM (Mw 125 kDa) (figure 2). The greatest reduction in TNF-α expression was found in Enz-GM pH 5.5 (Mw 0.79 kDa), with an average of 3557 pg/mL (figure 3). The decrease in iNOS production in both GM and Enz-GM suggests potential anti-inflammatory effects (figure 4). These findings are consistent with the study by Qiaoran Zheng et al. (2019), which indicated that degraded glucomannan, or glucomannan with a lower Mw, can inhibit the activity of IL-6, TNF-α, and iNOS in macrophages [23]. The main difference between this study and the research by Qiaoran Zheng et al. is the method used for glucomannan degradation. While Zheng et al. applied ultrasonic degradation, this research employed an enzymatic method. Additionally, a study by Mohammad Affandi et al. [24] found that porang's glucomannan (PGM) and its hydrolysates (PGMH) exhibited stronger NO inhibitory activity compared to the positive control, with PGMH showing the highest potential among the treatments.

5. CONCLUSION

Differences were observed in the cytokine profiles secreted by RAW 264.7 macrophage cells in Enz-GM, with a reduction in IL-6, TNF-α, and iNOS expression at Enz-GM pH 9 (Mw 1.24 kDa). However, TNF-α expression was more pronounced at Enz-GM pH 5.5 (Mw 0.79 kDa). Statistical analysis showed that the expression of the pro-inflammatory cytokines IL-6 and iNOS was most evident at Enz-GM pH 9 (Mw 1.24 kDa), and both were significantly different ($p \leq 0.05$) compared to GM. However, there was no significant difference in TNF-α expression when compared to GM.

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