The Immunostimulant Activities of the Gambir (*Uncaria gambir* Roxb) on Raw 264.7 Cell

Elsya Gustia[1], Yufri Aldi[1], Dira Hefni[1], Sefrianita Kamal[2], Dachriyanus[1], Fatma Sri Wahyuni[1*]

[1]*Faculty of Pharmacy, Universitas Andalas, Kampus Limau Manis, 25163, Padang, West Sumatra, Indonesia*
[2] *Pharmacy Study Programme, Universitas Dharma Andalas Padang, West Sumatera, Indonesia.*

[*]*Corresponding author. Email: fatmasriwahyuni@phar.unand.ac.id*

ABSTRACT

Gambir (*Uncaria gambir* Roxb) is a plant that is known to have various activities, one of which is immunostimulant. This study aims to determine whether gambir from Payakumbuh affects the viability and immunostimulant activity on RAW 264.7 cells induced by lipopolysaccharide (LPS). This study was conducted in vitro with a concentration 1, 10, 100µg/mL on the viability and immunostimulatory activity of RAW 264.7 cells. Cell viability testing using the microtetrazolium (MTT) method aims to see whether the gambir sample used is safe and not toxic on RAW 264.7 cells by looking at the cell viability value >90%. Gambir 1,10, 100µg/mL was safe and non-toxic to RAW 264.7 cells with a viability value >90%. Thus, the concentration of gambir used can be further tested, namely the immunostimulant activity of gambir on RAW 264.7 cells induced by LPS looking at the levels of IL-6 (proinflammatory cytokines) compared with LPS as a control using the sandwich ELISA (Enzyme-Linked Immunosorbent Assay). It was found that gambir 1,10, 100µg/mL could significantly increase the levels of IL-6 ($p<0,05$) which was tested with one-way ANOVA and continued with Duncan's post hoc test. Based on these results, it can be concluded that gambir from Payakumbuh (*Uncaria gambir* Roxb) is safe and non-toxic and has immunostimulant activity by increasing the levels of IL-6 which were tested on RAW 264.7 cells induced by LPS.

Keywords: *Uncaria gambir* Roxb, *RAW 264.7 cell, cell viability, immunostimulant*

1. INTRODUCTION

Gambir is a tropical plant belonging to the Rubiaceae family with a height of about 1,5-2 meters which grows mostly in the highlands of Argentina, Philippines, and Indonesia [1]**.** Gambir is a plant native to Southeast Asia, especially the island of Sumatra. Grows in open areas in the forest, humid forest areas, open areas of former cultivation or forest edges at an altitude of 200-900 m above the sea level [2].

Gambir has various benefits such as antioxidant, anti-inflammatory [3], antiseptic [4], antihypercholesterolemic [5], a mixture of drugs, for burns, headaches, diarrhea, dysentery, thrush medicine, skin pain medicine [6], raw materials In the pharmaceutical industry [1] gambir extract has also been studied showing that it can increase the activity and ability of macrophage cells to phagocytose Staphylococcus epidermis which was tested in vivo in male rats at concentrations of 0,1, 1, 10, 100, and 1000 ppm with one ANOVA data analysis direction [7].

In the non-specific immune system, macrophage cells will release a number of pro-inflammatory cytokines such as interleukins (IL-12, IL-1β, IL-6), and tumor necrosis factor alpha (TNF-α). IL-6 is included in a group of pro-inflammatory cytokines so that it can be used as an indicator of the level of inflammation that is pleiotropic and multifunctional which plays an important role in body defense, immune response, nerve cell function and hematopoiosis [8]. The presence of macrophage activation is also an early stage of transfer of bioinformation to produce immunomodulatory activity [9].

Immunomodulator is a substance or compound that can modulate the activity and function of the body's immune system by dynamically regulating immune cells such as cytokines [10]. Immunomodulators are divided into three groups, namely immunostimulators that function to increase the activity and function of the immune system, immunoregulators that can regulate the immune system, and immunosuppressors to inhibit or suppress immune system activity [11].

This study aims to observe the immunostimulant activity of gambir from Payakumbuh. This study also examines the

cytotoxicity of Payakumbuh gambir against 264.7 RAW cell line which is a preliminary study to see a safe and non-toxic dose for further testing of immunostimulant activity.

RAW 264.7 cells are moncocyte-macrophage cell lines that are widely used in research on the immune system because they are similar to macrophages produced by bone marrow. RAW 264.7 cells were derived from male mouse ascites tumor induced by intraperitoneal injection of A-MuLV (Abelson Leukemia Virus). Raw 264.7 cells can synthesize and secrete lysozyme, phagocytize zymosan, mediate antibody-induced lysis and can phagocytize sheep erythrocytes [12].

2. METHODS

2.1. Plant material and chemicals

Uncaria gambir Roxb aqueous extract were obtained from Payakumbuh, Indonesia. RAW 264.7 cells (ATCC®TIB-71TM), Dulbecco's Modified Eagle Medium (DMEM) (Gibco), fetal bovine serum (FBS) (Gibco), dimethyl sulfoxide (DMSO), penicillin-streptomycin 2% (v/v) (Gibco), trypsin-EDTA 0,25% (Gibco), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetra zolium bromide (MTT) (Sigma), Phospate Buffer Saline (PBS), lipo-polysaccharide (LPS) (Sigma), Mouse IL-6 ELISA kit (Bio Technology Laboratory®).

2.2. Cell culture

RAW 264.7 cells were grown in DMEM ((Dubelcco's Modified Eagle Medium) with supplementation of 10% FBS and 1% penicillin-streptomycin. Cell cultures were incubated at 37ºC in a humidified atmosphere and 5% CO2 until cells were confluent, then cells were harvested with trypsin-EDTA [13].

2.3. Cell viability assay

Cell viability assayt using the MTT method to determine the sample concentration that is safe and non-toxic on RAW 264.7 cells. MTT is a sensitive, quantitative and reliable colorimetric method for measuring cell viability, proliferation and activity. Based on the ability of living cells to reduce MTT salts which are yellow and soluble to purple formazan [14].

Add as much as 180 µL of the prepared and calculated cell suspension (amount and volume measured) into each plate 96 well except for blank wells which only contain medium. Then incubate for 24 hours at 37º C, 5% CO2. Then, add 20 µL of the prepared sample solution into each of the sample wells on a 96-well plate containing the previous cell suspension. The control well was only filled with 180 µL of cell suspension, and the blank was only filled with 200 µL of medium. The plate was again incubated for 24 hours in an incubator for 24 hours at 37º C, 5% CO2. Observe the changes that occur in the cells during the incubation period [15].

Pipette 20 µL of 2 mg/mL MTT solution into each well. Incubate for 3-4 hours at 37º C, 5% CO2. After 3-4 hours, a purple precipitate of formazan crystals will be seen. The supernatant was discarded, then the remaining supernatant was drained with a tissue. After leaving only the formazan crystal violet precipitate, dissolve the precipitate with 100 µL of DMSO in each well. Measure the absorption with a microplate spectrophotometer with a wavelength of 550 nm [15].

Absorbance data obtained from measurements can be determined by the percentage of cell viability using the following formula:

$$\%\ cell\ viability = \frac{A(sample\ cell - medium\ control)}{A(control\ cell - medium\ control)}\ x\ 100\ \%$$

2.4. Immunostimulant activity

Insert as much as 900µL of the cell suspension that has been made and counted (amount and volume measured) into each plate 24 wells except for blank wells which only contain medium. Then incubate in an incubator for 24 hours at 37º C, 5% CO2[16].

Transfer 100 µL of the sample solution into each of the sample wells on a 24-well plate containing the previous cell suspension. The normal control well was only filled with 900 L of cell suspension, and the blank was only filled with 900 L of medium. The plate was incubated again for 2 hours. After 2 hours, add the LPS solution into each well to obtain a concentration of 1 g/mL and incubate again for 24 hours. After that, the medium was taken and centrifuged at 2000 RPM for 20 minutes at 2-8ºC. The supernatant portion was taken for measurement of IL-6 levels by ELISA method [16].

2.5. Level measurement of IL-6

IL-6 cytokine levels were measured by the ELISA sandwich method using the ELISA kit. Add 50 L of the standard solution to the standard well and 40 L of the sample and 10 L of the IL-6 antibody to the sample well on the plate provided in the ELISA kit. Then add 50 L of streptavidin-HRP to the sample and standard wells, except for the blank wells. Mix well and cover with a seal and incubate for 30 minutes at 37º C. After that, open the seal and wash the plate 5 times with 0.35 mL of wash buffer for 30-60 seconds each wash [17].

Add 50 µL of substrate solution A to each well then add another 50 µL of substrate solution B. Incubate the plate

with the new seal for 10 minutes at 37º C in the dark. After that, add 50 µL of stop solution to each well, and the color changes from blue to yellow. And the value of optical density (OD) of each well can be read using a microplate reader at 450 nm within 10 minutes after adding the stop solution [17].

2.6. Statistical analysis

The data is expressed as the mean ± standard deviation (SD) of the results obtained from 3 repetitions of the one-way Analysis of Variance (ANOVA) with Duncan's test using SPSS statistical software. The p value < 0.05 indicates a significant difference.

3. RESULTS AND DISCUSSION

3.1. Cell viability assay

The viability of RAW 264.7 cells was carried out using the MTT (microtetrazolium) method, namely counting the number of living cells (viability) by looking at the absorbance value measured using a microplate reader with a wavelength of 550 nm from the reaction of the MTT reagent with the dehydrogenase enzyme produced by the cells. Metabolically active cells produce purple formazan crystals. The number of formazan crystals produced is proportional to the number of living cells and is also proportional to the absorbance value produced. So that the darker the purple color produced, the higher the absorbance value and the higher the viability of the cell. The advantages of the MTT method are that the test is quite sensitive, fast, semi-automatic, and can measure a large number of samples at one time [15].

Viability assay need to be carried out to determine the effect of the gambir sample used on the viability of RAW 264.7 cells and to determine the safe and non-toxic concentration of gambir to be used in the next test, namely the immunostimulant activity of gambir against RAW 264.7 cells.

Cell viability was calculated for each concentration with 3 repetitions. The sample is said to be safe and non-toxic to cells if the cell viability value is above 90% (44). It was found that gambir was safe and non-toxic to RAW 264.7 cells with cell viability at concentrations of 1, 10, and 100µg/mL was 121,866; 125,167; 136.088% (Table 1) with p < 0,05. From the cell viability obtained, it is known that gambier can also increase the proliferation of macrophages.

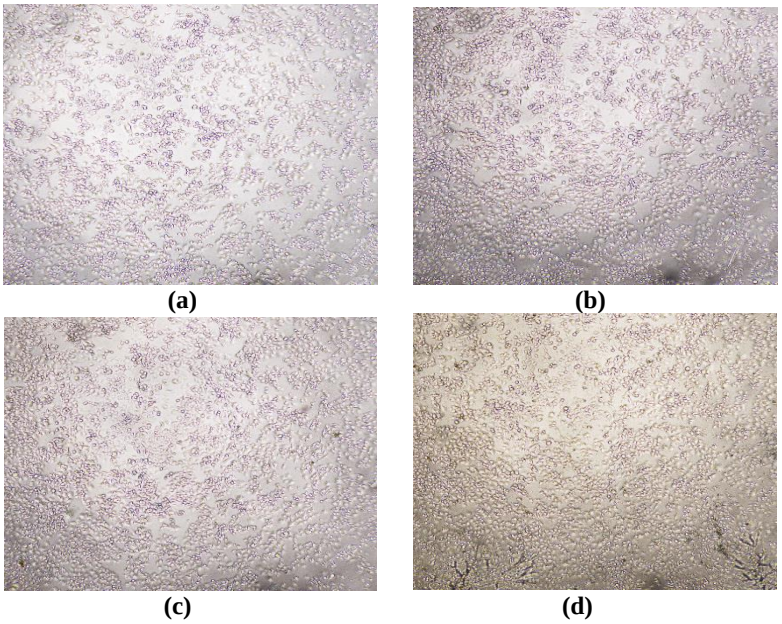

Figure 1. Normal RAW 264.7 cell (a); RAW 264.7 cells treated with gambir 1µg/mL(b); RAW 264.7 cells treated with gambir 10µg/mL(c); RAW 264.7 cells treated with gambir 100µg/mL(d), All images are magnified at 40X in inverted microscope

Table 1. Percentage of cell viability

Gambir (µg/mL)	Absorbance			Cell viability (%)			Mean ± SD
	1	2	3	1	2	3	
1	1,688	1,663	1,673	122,859	120,998	121,742	121,87 ± 0,94
10	1,773	1,735	1,649	129,188	126,359	119,955	125,17 ± 4,73
100	1,892	1,885	1,820	138,049	137,528	132,668	136,09 ± 2,96

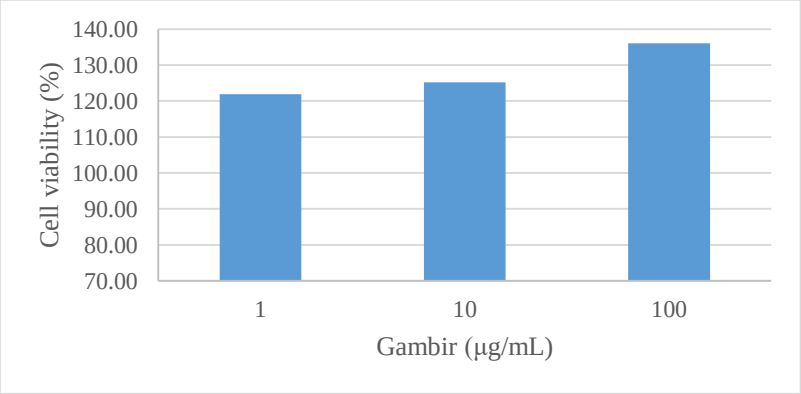

Figure 2. Bar chart of relationship between gambir concentration and % viability of RAW 264.7 cells

3.2. Immunostimulan activity

The use of sandwich ELISA is specifically for measuring antigen levels. Also, because it uses 2 antibodies the use of sandwich ELISA is more sensitive than other types of ELISA so it is 2-5 times more precise and accurate [18].

This study used cytokines from RAW 264.7 cells with LPS (inducing) as a control, RAW 264.7 cells without treatment (untreated), and RAW 264.7 cells added with gambir solution with LPS as a sample. LPS is a bacterial endotoxin found in the outer membrane of the cell wall of gram-negative bacteria. When LPS reacts with RAW 264.7 cells, the cells will phagocytize LPS which is a natural defense mechanism from foreign body attacks and then secrete pro-inflammatory cytokines which are an immune response [19].

ELISA results can be observed qualitatively and quantitatively. Qualitative results can be seen from the change in color to yellow which is the result of the reaction of antibodies with antigens. The higher the intensity of the yellow color produced, the higher the absorbance value and the more antigen-antibody bonding reactions that occur, which means the higher the number of tested antigens. Quantitative results can be assessed from the optical density measured using a microplate reader with a wavelength of 450 nm [18].

The standard ELISA equation is obtained from the optical density value of the standard solution which can then be determined the concentration of each sample which is calculated using My Curve Fit software using the 4PL curve. The 4PL curve is a regression model that is often used to analyze bioassays such as especially ELISA.

In immunostimulant activity, proinflammatory cytokines IL-6 produced by macrophages will act as antigens that will bind to antibodies on the ELISA sandwich, the more proinflammatory cytokines produced

In the IL-6 test, the untreated level was 1.367 ng/mL and the control level was 4.054 ng/mL. Gambir 1, 10, and 100µg/mL were 4.591; 5,619; 5,108ng/mL with a percentage increase of 13,246 respectively; 38,604; 25.999% (Table. 2).

Table 2. Absorbance data and IL-6 levels from untreated, control and gambir on RAW 264.7 cells

Group	Absorbance			IL-6 levels (ng/mL)			Mean ± SD
	1	2	3	1	2	3	
Untreated	0,108	0,114	0,116	1,280	1,403	1,444	1,38 ± 0,09
Control	0,211	0,226	0,245	3,613	4,016	4,560	4,06 ± 0,48
Gambir (1µg/mL)	0,247	0,249	0,243	4,620	4,680	4,501	4,6 ± 0,09
Gambir (10µg/mL)	0,279	0,275	0,281	5,654	5,516	5,724	5,63 ± 0,11
Gambir (100µg/mL)	0,259	0,262	0,268	4,990	5,086	5,281	5,12 ± 0,15

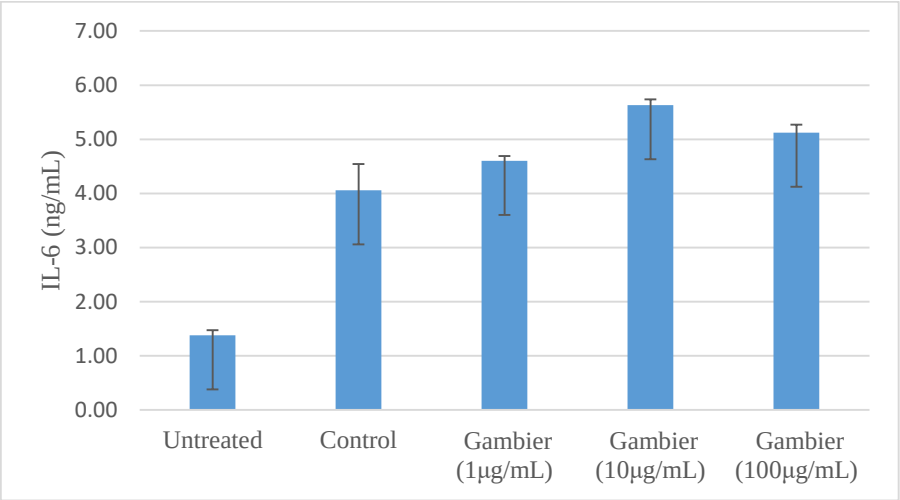

Figure 3. Effect of gambir on IL-6 production on RAW 264.7 cells induced by LPS

From the IL-6 levels data, it was found that gambir (Uncaria gambir Roxb) from Payakumbuh had immunostimulant activity which was characterized by increased levels of IL-6 compared to control on RAW 264.7 cells which are proinflammatory cytokines produced in the early phase of inflammation in phagocytosis. pathogens that enter the body. The more proinflammatory cytokines produced, the more the body's immune system will fight pathogens. Compounds that can increase the immune system can be called immunostimulants [11].

The presence of immunostimulant activity in gambir is due to the catechin content contained in it. Gambir is known to have the main compound catechin which is a flavonoid group. According to Devagaran & Diantini (2012) that flavonoids, curcumin, limonoids, and catechins can increase the activity of the immune system [19].

Increased levels of IL-6 due to flavonoids can induce NF-κB (Nuclear Factor-kappaB). NF-κB is a transcription factor that can induce the transcription of genes located in cells that will play a role in the immune response, cell proliferation and cell survival [20]. When NF-κB is activated, NF-κB will stimulate an immune response, one of which is the production of the proinflammatory cytokine IL-6 which then stimulates re-activation of NF-κB (feedback positive mechanism) so that the immune response can last longer. Thus, with the activation of NF-κB, the number of IL-6 cytokines produced also increases [21] [22].

However, at 100µg/mL gambir which is a large dose, it starts to showed decreased levels of the cytokines IL-6 which should undergo enhancement. Decreased production of IL-6 cytokines at 100µg/mL gambir may occur because the mechanism of the gambir sample is not caused by cell

population in which cell viability tests have been carried out to see if the sample not to kill RAW 264.7 cells and to avoid false positives in the event of decreased cytokine production. The gambir cell viability of 100µg/mL was obtained, namely 136,088% which indicates that the 100µg/mL gambier sample is safe and not toxic on RAW 264.7 cells.

The ups and downs in the production of IL-6 cytokines also occur in research by Felipe (2016) in which the flavonoid compound naringenin (NGN; 4',5,7-thrihydroxyflavanone) at concentrations of 30, 100, 300 nM decreased of IL-6 levels, and at a concentration of 1000 nM actually increased [23]. Also on research Kang Yi Su (2014) showed the compound 3,4-dihydroxytoluene at the concentration of 1,25; 2.5; 5µM decreased and at a dose of 10µM actually increased of IL-6 levels [24].

The decrease in the production of IL-6 cytokines was due to the decrease in the number of cytokines produced due to the inhibition of NF-κB in stimulating the production of TNF-α and IL-6 which are proinflammatory cytokines [25].

4. CONCLUSION

The immunostimulant activity on RAW 264.7 cells showed that gambir (*Uncaria gambir* Roxb) could enhance immunostimulant activities through increasing proliferation of macrophage and stimulating IL-6 cytokines secretion.

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