Malaysian Journal of Chemistry, 2022, Vol. 24(4), 144-149
(Special Issue: *International Conference on Natural Products 2022*)

Immunomodulatory Effects of Combined Ethanolic Extracts of *Begonia medicinalis* and *Moringa oleifera* in Wistar Rats Infected with *Staphylococcus aureus*

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Previous studies have shown that the extracts of *Begonia medicinalis* and *Moringa oleifera* possessed immunomodulatory properties. This study aims to determine the effective dose of ethanolic extracts of *Begonia medicinalis* combined with *Moringa oleifera* in modulating macrophage phagocytotic activity and interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) cytokine levels in rats infected with *Staphylococcus aureus*. The test animals were randomly divided into 5 groups. The negative control group was given carboxymethyl cellulosa sodium (CMC-Na), positive control group was given stimuno®, and treatment groups were given a combination of *B. medicinalis* and *M. oleifera* ethanolic extracts with doses of 100:100, 200:100 and 300:100 mg/kg BW, respectively. On the eighth day, the rats were sensitized with *Staphylococcus aureus* by intraperitoneal injection. After one hour, the peritonial fluid was taken from the peritoneal cavity to determine macrophage phagocytotic activity and blood was taken from intracardiovascular section to measure IFN-γ and TNF-α levels. The combined extracts of 100:100 mg/kg BW exhibited the highest percentage of phagocytotic activity (88.63% ± 5.22), IFN-γ (74.18±6.75 pg/mL) and TNF-α levels (15.83±1.48 pg/mL) compared to other treatments. Further statistical analysis showed the significant difference on each parameter between combined extract of 100:100 mg/kg BW and negative control ($p<0.05$). As conclusion, the combined extract of 100:100 mg/kg BW has activity as an immunomodulator based on macrophage phagocytotic activity and IFN-γ/TNF-α cytokine levels.

Key words: *Begonia medicinalis*; *Moringa oleifera*; phagocytotic macrophage; IFN-γ; TNF-α; immunomodulators

Received: September 2022; Accepted: November 2022

Recently, the environmental conditions with high pollution, erratic weather, use of drugs, radiation, unhealthy eating patterns, lack of exercise and high levels of stress become the factors in the decreasing of body's immunity that lead to the sensitivity of bacterial infection that causes the death of million people in the world, especially in developing countries (1). Therefore, the immunomodulators drugs, that can modulate the function and activity of the immune system, can be used as an alternative treatment to control the infection by increasing the immune system (2). Clinically, immunomodulators are used in patients with impaired immunity such as infections (3). Synthetic drugs that are commonly used to restore the balance of the immune system include immuno-suppressant drugs (azathioprine and chlorambuci) and immunostimulant drugs (isoprinosine, levamisole, and arginine)(4). These drugs work by stimulating the immune response through phagocytosis, complement system, antibody secretion, interferon release, T and B lymphocytes, synthesis of specific antibodies and cytokines (4). However, several side effects were reported such as the increasing of uric acid levels, agranulocytosis, urticaria, hepatotoxicity, and digestive tract disorders (5). Commonly, the drugs derived from nature have fewer side effects compared to synthetic drugs (6). Therefore, the drugs that comes from plants is needed as an alternative immunomodulators to minimize the side effects. Plants that have been reported as immunostimulator agents are *B. medicinalis* and *M. oleifera*.

B. medicinalis, known locally as benalu batu, has been traditionally used by the Wana tribe in the North Morowali regency, Central Sulawesi Province, Indonesia, to treat various diseases such as tumors, cancer, asthma, dry cough, and gout (7). The methanol extract, n-hexane fraction, ethyl acetate and water fraction of *B, medicinalis* have been proven to increase lymphocyte cell proliferation and act as immuno-stimulator with the highest stimulation index at the concentration of 100 g/mL (8). The ethanolic extract

†Paper presented at the International Conference on Natural Products 2022 (ICNP 2022)

of *B. medicinalis* herb at a dose of 60, 120 and 240 mg/Kg BW could increase the phagocytotic activity and stimulated the IFN-γ and TNF-α cytokines induction (9). Meanwhile, *M. oleifera* leaf extract exhibited immunostimulatory activity against hematopoetic stem cell (HSC) in mice infected with *Salmonella typhi* at lower doses of 14 and 42 mg/kg BW and at higher doses of 84 mg/kg BW, the extract demonstrated the immunosuppressant activity (10). Furthermore, the ethanolic extract of *M. oleifera* at a dose of 250 mg/kg BW had an increasing effect on the phagocytotic activity of male mice with the highest phagocytosis index (0.027) compared to other groups (11). The oral administration of 70% ethanol extract of *M. oleifera* leaves can increase the phagocytotic activity of macrophages in white mice where the highest activity was achieved at a dose of 100 mg/kg BW (12). Thus, this study aimed to investigate the immunomodulatory effects of combined ethanolic extracts of *B. medicinalis* and *M. oleifera* leaves on phagocytotic activity of macrophages, and IFN-γ and TNF-α cytokines production from *S. aureus*-infected rats.

EXPERIMENTAL

Plants Materials

B. medicinalis were obtained from the village of Toddopoli, Soyojaya District, North Morowali Regency and *M. oleifera* were collected from Sibedi village, Marawola District, Sigi Regency, Central Sulawesi Province, Indonesia. The collection of plants was carried out on February 2022. *B. medicinalis* was identified by Wisnu H Ardi (Taxonomist) from Bogor Botanic Garden, Indonesia and *M.oleifera* was identified by Sahlan (Biologist) from Celebense Herbarium, Tadulako University. Both plants were deposited with the voucher specimen code BSP 00020414 and MO00020415 at Phytochemistry Laboratory, Department of Pharmacy, Tadulako University.

Chemicals and Materials

Staphylococcus aureus ATCC 25923, aquadest, 96% ethanol (Mercks®), 0.5% CMC-Na (Mercks®), IFN-γ Kit (ABclonal®), TNF-α Kit (ABclonal®), ketamine®, Plate Count Agar (Mercks®), methanol, phosphate buffered saline (PBS) pH 7.8, physiological NaCl, immersion oil, 10% Giemsa dye, nutrient agar (NA), and stimuno® purchased from the local market.

Animals

The experimental animals used in this study were 25 male Wistar rats aged 10-12 weeks, weighing 150-250 grams, and in good health. The animal were acclimatized for 7 days with standard rodent food and given water *ad libitum.* The sample size used in this study is based on the Federer formula (13). The research procedure has followed the ethical approval obtained from the Medical and Health Research Ethics Committee, Faculty of Medicine, Tadulako University Number: 5458/UN 28.1.30/KL/2021.

Extraction

Leaves and stem of *B. medicinalis* (2.4 kg) and leaves of *M. oleifera* (2.1 kg) were washed and shade dried at room temperature. The dried plants were re-sorted, then chopped using scissor. Each dried plant was extracted by maceration method using 70% ethanol for 3x24 hours. The obtained filtrate was concentrated using a vacuum rotary evaporator at a temperature of 60-65ºC to obtain the crude extract of both plants (103.86 g of *B. medicinalis* and 175.83 g of *M. oleifera*).

Immunomodulator Activity Testing

The test animals were 25 male wistar rats devided in 5 groups. Group 1 as a negative control was given 0.5% CMC-Na, group 2 as a positive control was given Stimuno® containing commercial meniran (*Phyllantus niruri* Linn.) extract at a dose of 4.5 mg/kg BW, group 3, 4 and 5 were given a combined ethanolic extract of *B. medicinalis* and *M. oleifera* leaves at dose of 100:100, 200:100 and 300:100 mg/kg BW, respectively. The treatments on the test animals were carried out for 7 days by oral administration with the volume based on the weight of the experimental animals. On the eighth day, each rat was infected intraperitoneally with 0.5 mL of *Staphylococcus aureus* bacteria suspension, then left for 1 hour. Rats were anesthetized by using ketamine at a dose of 6.5-13 mg/kg BW, then the abdomen was dissected using a scalpel and sterile tweezers. If the peritoneal fluid in the rat's stomach was found in small quantities, then 1-2 mL of phosphate buffered saline (PBS) steril solution with pH 7.8 was added and shaken slowly. The peritoneal fluid was taken from the peritonial cavity by using 1 mL syringe. Peritoneal fluid was stained on the glass slide, fixed with the addition of methanol for 5 minutes, stained with 10% Giemsa stain, left for 20 minutes and then rinsed with running water. After the glass slide dried, the sample was dripped with immersion oil and viewed under a microscope (Olympus CX23 LED, Olympus) using magnification of 1000× (3). Meanwhile, the blood was also taken from each animal via intracardiac section and put into eppendorf tube that contain EDTA. Blood was centrifuged with a centrifuge (Series C2®) for 15 min at 3000 rpm to collect plasma. The collected plasma was then put into a microtube and stored in a container (-20°C) to be tested for IFN-γ and TNF-α levels using an ELISA Reader at a wavelength of 450 nm according to manufacturer's instructions. The results were expressed as picograms of cytokine per milliliter of protein (9,14).

Statistical Analysis

The research data were analyzed using SPSS (Statistical

Product and Service Solution) version 26. The phagocytotic activity of macrophages and TNF-α level were analyzed by using one way ANOVA test, followed by Post Hoc Tukey test, Meanwhile, the IFN-γ level was analyzed by using the Kruskal-Wallis test, followed by the Mann-Whitney test. Analysis was performed with a significance value of 95% ($p \leq 0.05$).

RESULTS AND DISCUSSION

Macrophage Phagocytotic Activity

The observation of macrophage phagocytotic activity was performed by using Giemsa staining on microscope with 1000× magnificient. CMC-Na was used as a negative control because it has no pharmacological effect on test animals. It has also inert properties and can produce stable suspensions (15,16). The positive control is stimuno®, a herbal product that contains *P. niruri* extract. It has been reported that *P. niruri* extract can enhance the immunity. It contains *phyllanthin* and *hypophyllanthin* that have anti-inflammatory activity that can strengthen the immunity (16,17). The combined extract of *B. medicinalis* and *M. oleifera* of 100:100, 200:100 and 300:100 mg/kg BW were chosen based on the previous report regarding the effective dose of immunomodulatory activity of *B. medicinalis* and *M.oleifera* leaves (9,12)

Active macrophages are characterized by the shape and size of macrophages that increase in size with highly variable extension of pseudopods, while inactive macrophages have smaller sizes and shapes than active macrophages (18). The difference between active and inactive macrophages can be seen in Figure 1. The value of macrophage phagocytotic activity can be calculated from the amount of active macrophages that carry out phagocytosis devided by the total number of cells and expressed in percent. The highest average percentage of macrophage phagocytotic activity in male Wistar strain rats was found from the combined extract of 100:100 mg/kg BW (88.63%± 5.22), compared to combined extract of 200:100 and 300:100 mg/kg BW, negative control (CMC-Na) and positive control (stimuno®), with the percentage of macrophage phagocytotic activity of 45.54%±12.72, 30.51%±23.64, 28.45%±17.06, and 56.43%±12.57, respectively. The post hoc Tukey test showed a significant difference between combined extract of 100:100 mg/kg BW and other treatment groups with $p < 0.05$ (Table 1).

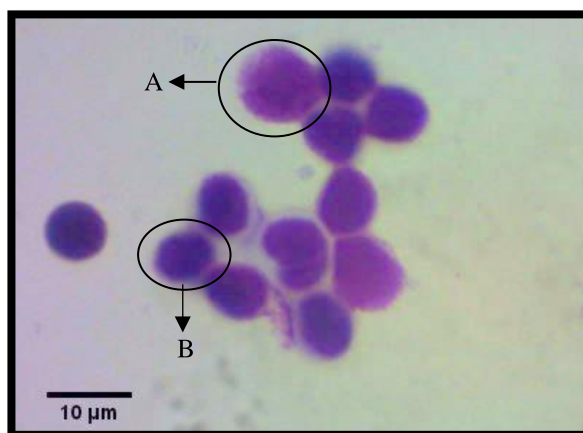

Figure 1. Peritoneal fluid smear with 1000x magnification (A) Active macrophages and (B) Inactive macrophages

Table 1. The percentage of macrophage phagocytotic activity after 7 days of treatment with combined extract of *B. medicinalis* and *M. Oleifera*

Test Group	Mean (%) ± SD
Negative Control	28.45±17.06[a]
Positive Control	56.43±12.57[ab]
1:1	88.63±5.22[b]
2:1	45.54±12.72[a]
3:1	30.51±23.64[a]

Note: Data are presented as mean ± SD of three observations. The different subscript letters in the table indicate a significant difference between the treatment groups with a 95% confidence level ($p<0.05$) using post hoc Tukey's test. 1:1= combined extract of 100 : 100 mg/kg BW, 2:1= combined extract of 200 : 100 mg/kg BW, and 3:1= combined extract of 300 : 100 mg/kg BW.

Table 2. Cytokine levels of IFN-γ and TNF-α after treated by combined extract of *B. medicinalis* and *M. oleifera* for 7 days.

Test Group	IFN-γ (pg/mL) ± SD	TNF-α (pg/mL) ± SD
Negative Control	18.88 ± 3.97^{a}	3.53 ± 2.92^{a}
Positive Control	49.18 ± 6.12^{b}	$12.37 \pm 0.79^{a,b}$
1:1	74.18 ± 6.75^{c}	15.83 ± 1.48^{b}
2:1	$21.91 \pm 11.57^{a,d,e}$	$7.50 \pm 3.86^{a,b}$
3:1	$25.54 \pm 5.46^{a,e}$	3.65 ± 3.82^{a}

Note: Data are presented as mean ± SD of three observations. The different subscript letters in the table indicate a significant difference between the treatment groups with a 95% confidence level ($p<0.05$) using post hoc Tukey's test for TNF-α and Mann-Whitney test for IFN-γ. 1:1= combined extract of 100:100 mg/kg BW, 2:1= combined extract of 200:100 mg/kg BW, and 3:1= combined extract of 300:100 mg/kg BW.

Cytokine Levels of IFN-γ and TNF-α

The measurement of IFN-γ and TNF-α levels were also in accordance with the percentage of macrophage phagocytotic activity. The highest levels of IFN-γ were also found in the combined extract of 100:100 mg/kg BW (74.18±6.75 pg/mL), compared to negative control, positive control, combined extract of 200:100 and 300:100 mg/kg BW which respectively had IFN-γ levels of 18.88±3.97 pg/mL, 49.18±6.12 pg/mL, 21.91±11.57 pg/mL, and 25.54±5.46 pg/mL. Meanwhile, the measurement of TNF-α level is also in line with IFN-γ levels. The highest TNF-α level was found in combined extract of 100:100 mg/kg BW (15.83 ±1.48 pg/mL) compared to the combined extract of 200:100 mg/kg BW, 300:100 mg/kg BW, negative control and positive control, which had an average of 7.50±3.86 pg/mL, 3.65±3.82 pg/mL, 3.53±2.92 pg/mL and 12.37±0.79 pg/mL, respectively. Further statistical analysis showed the significant difference of combined extract 100:100 mg/kg BW with other treatments ($p<0.05$) (Table 2). This showed that the combined extract of 100:100 mg/kg BW of *B. medicinalis* and *M. oleifera* has activity as an immunomodulator because it can increase the production of IFN-γ and TNF-α cytokine levels.

Macrophages can be activated through several types of stimuli such as microbes and their products, antigens, dead cells, T lymphocyte membrane proteins, or cytokines. The activated macrophages produce cytokines such as TNF-α, interleukin-1 (IL-1), and interleukin-6 (IL-6) which will trigger acute inflammation. The inflammation will attracts monocytes and neutrophils from the blood vessels to the site of infection. Macrophages also produce IL-12 which stimulates the differentiation of CD4+ cells into T helper 1 (Th1) effector cells that produce IFN-γ. IFN-γ is a potent mediator in macrophage activation. In addition, IFN-γ can stimulate B cells to produce IgG which acts as an opsonin that can increase the phagocytotic activity of macrophages (19). TNF-α is the main cytokine in the acute inflammatory as response to gram-negative bacteria and other microbes. Severe infections can trigger the production of large amounts of TNF-α which causes a systemic reaction. The main sources of TNF-α are mononuclear phagocytes and antigen-activated T cells, NK cells, and mast cells. Lipopolysaccharides are potent stimuli for macrophages to secrete TNF-α. IFN-γ produced by T cells and NK cells also stimulates macrophages, including increasing TNF-α synthesis (20). TNF-α has biological effects such as mobilizing neutrophils and monocytes to the site of infection and activating these cells to get rid of microbes and stimulating the hypothalamus to induce heat called endogenous pyrogens (21).

Some studies reported the presence of flavonoids and saponins from *B.medicinalis* (7,22–24). While ethanol extract of *M. oleifera* was reported to contain alkaloids, flavonoids, tannins/phenolics, saponins and steroids (25,26). Compounds that play a role in enhancing the immune system are flavonoids, saponins and alkaloids. These compounds play a role in triggering the up-regulation of helper T cells by increasing the production of interleukin-12 (IL-12) that required by CD4+ T cells to influence lymphocyte proliferation which causes Th-1 cells to be activated. Activated Th-1 cells will produce IFN-γ that activate macrophages characterized by the increasing of phagocytotic activity. This mechanism is more efficient in killing antigens (27–29). Our previous research showed that the immunomodulatory properties of *B.medicinalis* ethanolic extract tend to stimulate the immune response by dose-dependent activity (9). However, when it is combined with *M.oleifera* leaves ethanol extract, the high potency of immunomodulatory activity of *B. medicinalis* was obtained at the lowest concentration (100 mg/kg BW). The presence of flavonoids in the extracts of *B. medicinalis* and *M oleifera* leaves allows to work either as an immunostimulant or immunosuppressant. If the activity of the immune system is reduced, then flavonoids will send intracellular signals to cell receptors to increase their activity. Conversely, if the immune system works excessively, then flavonoids will reduce the work of the immune system. So, the flavonoids in both extracts may have function as a balancer for the immune system (30). Moreover,

flavonoids also have effects on suppresing the immune system that leads to inhibit macrophages activation at the optimum concentration (31,32).

CONCLUSION

The combined ethanolic extract of *B. medicinalis* and *M. oleifera* leaves has an immunomodulatory effect based on macrophage phagocytotic activity and cytokines levels of IFN-γ and TNF-α. The highest immunomodulatory activity was found at a combined dose of extracts of 100:100 mg/kg BW. Therefore, it is suggested to be further developed as raw material for herbal drug formulation.

ACKNOWLEDGEMENTS

Authors acknowledge The General Directorate of Higher Education, Research and Technology, The Ministry of Education, Culture, Research and Technology, Republic of Indonesia, for the financial support through National Competitive Applied Research Grant year 2022 (No. 877.f/UN28.2/PL/2022)

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