

Immunomodulatory Activity of *Begonia Medicinalis* Ethanolic Extract in Experimental Animals

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ABSTRACT

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Benalu batu (*B. medicinalis*) is a plant endemic to Central Sulawesi and has been reported to possess anticancer, antioxidant, and antiviral properties but the mechanism of action for these activities is still unclear. Therefore, this study aims to evaluate the immunostimulatory effect of *B. medicinalis* ethanol extract in an *in-vivo* model by measuring the macrophage phagocytotic activity and cytokine production of tumor necrosis factor-alpha (TNF- α) and interferon-gamma (IFN- γ) in male mice and rats, respectively. Further determination of total saponin, phenolic, and flavonoid contents using the spectroscopic method was also performed. The extract was obtained by the maceration method for 3 \times 24 h using 70% ethanol with doses of 60, 120, and 240 mg/kg body weight (bw). The percentage of macrophage phagocytosis and the TNF- α , and IFN- γ levels were measured on the eighth day, one hour after intraperitoneal injection of *Staphylococcus aureus* ATCC 25923. The result showed that ethanol extract of *B. medicinalis* activated macrophage phagocytosis in a dose-dependent manner, with a significant increase in cytokine expression of TNF- α and IFN- γ . The optimal dose was 240 mg/kg bw, with a higher percentage of phagocytic activity and higher levels of TNF- α and IFN- γ than Stimuno[®] as a positive control and other dosage treatments. Meanwhile, the total saponin, phenolic, and flavonoid contents were found to be 319.72 \pm 26.46 mg EE/g, 112.02 \pm 17.16 mg GAE/g and 11.35 \pm 1.06 mg QE/g extract, respectively. Based on the results, *B. medicinalis* ethanol extract can act as an immunomodulator with a high concentration of total saponin, total phenolic as well as total flavonoid contents, and these results provide a scientific basis for traditional usage of this herb plant.

Keywords: *Begonia medicinalis*, TNF- α , IFN- γ , Phagocytosis, Macrophage

INTRODUCTION

The recent global pandemic caused by SARS-CoV-2 and other viral diseases, as well as the quest for better and more effective medications, have prompted the search for therapies from natural sources based on immune response mediation. The immune system is the organism's defense mechanism which prevents and eliminates pathogens/antigens that are potentially harmful. It can be grouped into the innate and adaptive immune systems (Fristiody *et al.*, 2020). Furthermore, immune responses are enhanced by

certain compounds called immunomodulators which affect the humoral and cellular immune system, either by increasing or suppressing the function. Immunostimulatory agents have been broadly applied to fight cancer, bacterial and viral infections, as well as immunodeficiency diseases. Meanwhile, immunosuppressive agents are used to treat autoimmune diseases and in organ transplantation (Block & Mead, 2003; Catanzaro *et al.*, 2018; Chaplin, 2010). Bacterial and viral infections are often associated with the immune

response, while immunostimulatory agents enhance the body's defense against pathogens. Cancer is also connected with increased levels of immune system cells such as T and β cells, as well as macrophages (DeNardo & Coussens, 2007; Labro, 2012; Qu *et al.*, 2018; Wang *et al.*, 2019).

Immunostimulants are characterized by macrophage phagocytic activity stimulated by cytokines such as IFN- γ , TNF- α , interleukin (IL)-1, and IL-6. IFN- γ is produced endogenously by activated CD⁴⁺ T lymphocytes as well as natural killer (NK) cells and is the main cytokine that plays a role in macrophage activation cells, mononuclear phagocytes, antigen-activated T cells, and macrophages. After production, these cytokines activate neutrophils, monocytes, and other macrophages to carry out phagocytosis and play a role in regulating macrophages to produce IL-12 which increases the production of IFN- γ by NK cells. TNF- α and IFN- γ are also known as pro-inflammatory cytokines due to their connection to macrophage phagocytosis (Kany *et al.*, 2019).

One of the medicinal plants that can be used as an immunomodulator is benalu batu (*Begonia medicinalis*). This plant is endemic to Central Sulawesi and has been reported to increase lymphocyte cell proliferation (immunostimulant) with no suppression effect on lymphocyte cells (Khumaidi *et al.*, 2020). Moreover, it also possesses anticancer, antioxidant, and antiviral properties (Anam *et al.*, 2014; Zubair *et al.*, 2021). Saponin (steroidal glycoside) of β -sitosterol-3-O- β -D-glucopyranoside, 2-O- β -glucopyranosil cucurbitacin D, and 9(11) α -16(17) α -dioxirane, 20,25-dihydroxy- β -sitosterol-3-O- β -glucopyranoside have been reported to exhibit anticancer activity by inhibiting the proliferation of cancer cell lines in vitro (Zubair *et al.*, 2021; Zubair *et al.*, 2020). Based on the practical use of this herb as an anticancer medicinal plant, supported by in vitro data for its potency as an anticancer, antiviral, and immunostimulant agent, this study aims to evaluate the immunomodulatory properties of *B. medicinalis* ethanol extract in relation to the macrophage phagocytotic activity activated by cytokine production of TNF- α and IFN- γ . The total saponin, total phenolic and total flavonoid contents in the extract were also evaluated.

MATERIAL AND METHODS

Materials

Benalu batu (*Begonia medicinalis*) was obtained from Toddopoli village, Soyojaya district, North Morowali, Central Sulawesi Province, and the

plant species was determined at LIPI Bogor Botanical Gardens. The Rat TNF- α ELISA kit (RK00029-96well) and Rat IFN- γ ELISA kit (RK00199-96well) were purchased from Abclonal®. *Staphylococcus aureus* ATCC 25923 (MBRIO) was prepared as a bacterial suspension and standardized at 0.5 McFarland (equivalent to a concentration of 10⁸ CFU/mL), Stimuno® purchased from drug store in Palu, ethanol p.a, PBS (Phosphate Buffered Saline) pH 7.2, 0.9% NaCl, Na-CMC, sodium alginate, calcium chloride (CaCl₂), chloroform, methanol, 10% Giemsa stain, immersion oil, 0.5% ketamine, vanillin, sulphuric acid, escin, folin-ciocalteu reagent, sodium carbonate, gallic acid, quercetine, aluminium chloride, potassium acetate, aquabidest and distilled water were all purchased from Sigma-Aldrich®.

Extraction

The herb *B. medicinalis* (leaf and stem) was dried at room temperature and then sorted manually. About 2 kg of sample was ground to obtain a powder, and then the powder was extracted by the maceration method with 70% ethanol at room temperature for 3 × 24 h. The maceration process was repeated three times. The liquid extract obtained was then evaporated with a rotary vacuum evaporator at 60–65°C to give a viscous extract (162.6 g). The extracts were freeze-dried and used for further analysis.

Animals

The animals (male mice and rats) were obtained from Research Centre Laboratory, Agency of Health Research and Development, Palu, Indonesia, housed under standard laboratory conditions, and fed with standard feed along with water ad libitum. The study was conducted based on institutional ethical guidelines issued by the Ethics Committee for Medical and Health Research, Faculty of Medicine, Tadulako University no. 7918/UN28.1.30/KL/2020 and 1933/UN28.1.30/KL/2021.

Macrophage Phagocytosis Activity Test on Mice (*Mus musculus*)

Phagocytosis activity tests of *B. medicinalis* ethanol extract were performed in male Balb/c mice. The animals were 20 male mice divided into five groups (n = 4), group 1 received 0.5% Na-CMC orally as a negative control, 2 Stimuno® at a dose of 6.5 mg/kg body weight (bw) as a positive control, 3 *B. medicinalis* extract at a dose of 60 mg/kg bw, 4

extract dose of 120 mg/kg bw, and 5 extract dose of 240 mg/kg bw. The treatment was carried out once a day for seven days orally, according to the volume of administration. On day 8, each mouse was injected intraperitoneally with 0.5 mL of a bacterial suspension of *Staphylococcus aureus* ATCC 25923, and then left for 1 hour before surgery. The mice were anesthetized with ketamine, and then the abdomen was dissected using a scalpel and sterile tweezers. When a small amount of peritoneal fluid was found in the abdomen, 1–2 mL of sterile PBS solution (pH 7.8) was added and slowly agitated. Next, 1 mL of peritoneal fluid was removed, smeared on a glass slide, and fixed with methanol for 5 minutes, then stained with 10% Giemsa stain. It was allowed to stand for 20 minutes, and rinsed with running water. After the slides were dry, a drop of immersion oil was added and the specimens were viewed under a microscope (Olympus CX23 LED, Olympus) at 1000x magnification (Wahyuni *et al.*, 2019). The phagocytic activity of macrophages in the peritoneal fluid was calculated as follows:

$$\% A = \frac{B}{C} \times 100\%$$

A=Phagocytic activity; B=number of active macrophages; C=number of observed macrophages

TNF- α and IFN- γ Level Assay on Rats (*Rattus norvegicus*)

The TNF- α and IFN- γ levels were measured in Wistar male rats divided into five groups (n = 4) and given treatment for seven days as follows: Group 1 was given Na-CMC 0.5% as a negative control, 2 Stimuno® at a dose of 4.5 mg/kg bw as a positive control, 3 *B. medicinalis* extract at a dose of 60 mg/kg bw, 4 extract dose of 120 mg/kg bw, and 5 extract dose of 240 mg/kg bw. On day 8, each animal was infected with 0.3 mL of *Staphylococcus aureus* ATCC 25923 and then allowed to stand for 1 hour. Surgery was performed, and blood was taken from the heart and placed in an EDTA tube. Subsequently, blood was centrifuged (C2 Series®) for 15 minutes at 3000 rpm to collect the plasma. The collected plasma was placed in a micro tube and stored in a container (20°C). The assay of TNF- α and IFN- γ levels was conducted using an ELISA Reader (Elabscience®) with the manufacturer's instructions. The results were expressed as picograms of cytokine per milliliter of protein (Fristiohady *et al.*, 2020).

Determination of total saponin

A total of 10 mg of extract was weighed and dissolved in 10 mL of ethanol p.a. Then, 250 μ L of the sample solution was added to 250 μ L of 8% vanillin (w/v) and 2.5 mL of sulfuric acid (72%). The tubes were closed, vortexed, and incubated in a water bath with a shaking speed of 100 rpm at 60°C for 15 minutes and then cooled for 5 minutes in ice water. After that, the absorbance of the mixture was measured with Cecil CE7410 UV-Vis spectrophotometer at a maximum wavelength of 599.5 nm. The treatment was carried out 3 times (triple). The absorbance result is then plotted against a standard curve of escin that prepared with the concentration of 10, 20, 40, 80 and 160 ppm. The results are expressed as milligrams of escin equivalent per gram of extract (mg EE/g) (Chua *et al.*, 2019).

Determination of total phenolic

A total of 10 mg of extract was weighed and dissolved in 10 mL of ethanol p.a. 1 mL of the extract solution was pipetted, added 0.4 mL of Folin-Ciocalteu reagent, then shaken homogeneously with a vortex and allowed to stand for 8 minutes. 4 mL of 7% Na₂CO₃ solution was added and then vortexed. 4.6 mL of aquabidest was added and allowed to stand for 2 hours at room temperature. The treatment was carried out in triplicate. After that, the absorbance of the mixture was measured on Cecil CE7410 UV-Vis spectrophotometer at a maximum wavelength of 750.5 nm. The absorbance value obtained was then plotted against a standard curve of gallic acid (concentration of 20, 30, 40 and 50 ppm) to obtain the total phenolic concentration. Yield is expressed in units of mg gallic acid equivalent per gram of extract (mg GAE/g) (Ahmad *et al.*, 2015; Sulastri *et al.*, 2018).

Determination of total flavonoid

A total of 10 mg of extract was weighed and dissolved in 10 mL of ethanol p.a. Then, 1 mL of the extract solution was pipetted with 0.2 mL of 10% (w/v) AlCl₃ solution, 0.2 mL of 1M potassium acetate and 5.6 mL of distilled water. The mixture was allowed to stand for 30min at room temperature in a dark room. Tests were carried out in triplicate. The absorbance was measured at a maximum wavelength of 433.5 nm. The absorbance value obtained was then plotted against the curve of standard quercetin (concentration of 10, 20, 30, 40 and 50 ppm) to obtain the total flavonoid content.

Results are expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g) (Aryal *et al.*, 2019; Zubair *et al.*, 2021).

Statistical analysis

The linear regression method was used to calculate the levels of TNF- α , IFN- γ , as well as the total saponin, phenolic, and flavonoid using SPSS 17.0 (SPSS, Inc, Chicago IL, USA). Further tests were then carried out through the one-way ANOVA and post hoc LSD analysis.

RESULT AND DISCUSSION

The immunomodulatory properties of *B. medicinalis* ethanol extract were assessed by measuring the macrophage phagocytotic activity and cytokine production of TNF- α and IFN- γ levels in male balb/c mice and Wistar albino rats, respectively. The extracts were administered for seven consecutive days orally to increase non-specific immune responses (Wahyuni *et al.*, 2019), along with Stimuno[®] as a positive control. Stimuno[®] is a herbal immunomodulatory agent containing extracts of *Phyllanthus niruri*. Extract doses of 60, 120, and 240 mg/kg bw were selected for the treatment based on the conversion calculation of the IC₅₀ value of lymphocyte proliferation cell activity reported previously by Khumaidi *et al.* (2020). Each mouse was injected with a suspension of *Staphylococcus aureus* ATCC 25923 bacteria intraperitoneally on the eighth day of treatment. This exposure will trigger the infection in animals because *S. aureus* contains lipoteichoic acid (LTA) that affects the immune response to activate the expression of cytokines such as IFN- γ and TNF- α (Fournier & Philpott, 2005).

Effect of *B. medicinalis* ethanol extract on macrophage phagocytosis

Macrophage phagocytotic activities after extract administration for seven consecutive days (Figures 1 and 2). Based on the results, the immunomodulatory effect of the extract was dose-dependent. The extract increased the percentage of phagocytotic activity from 85.66% (60 mg/kg bw) to 90.20% (240 mg/kg bw). This effect was higher than that of Stimuno[®] as a positive control. In addition, the Post hoc LSD analysis showed a significant difference for each treatment group with $p < 0.05$.

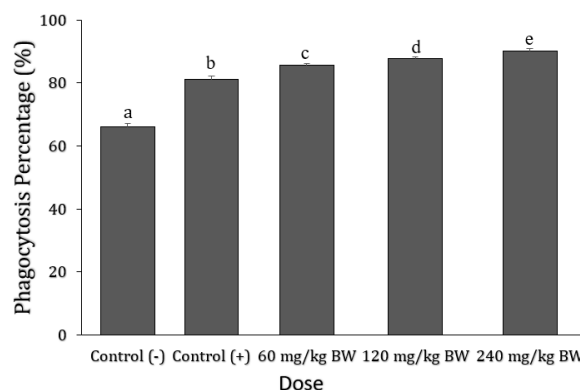


Figure 1. Macrophage phagocytotic activity (%) against *Staphylococcus aureus* after 7 days of treatment with *B. medicinalis* ethanol extract. Data are presented as mean \pm SD of four observations. The means of the different letter superscript are significantly difference between each group by post hoc LSD test at 0.05 significance level.

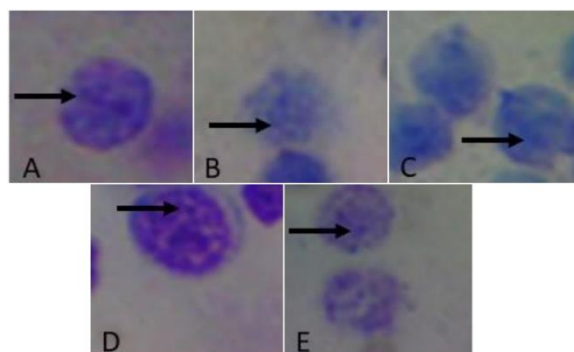


Figure 2. Phagocytosis of macrophage cells, treatment with negative control (A), positive control (B), *B. medicinalis* extract 60 mg/kg BW (C), *B. medicinalis* extract 120 mg/kg BW (D), *B. medicinalis* extract 240 mg/kg BW (E). Black arrows showed *S. aureus* in the macrophage

Effect of *B. medicinalis* ethanol extract on cytokine production of TNF- α and IFN- γ

The extract also had a dose-dependent effect on the production of the cytokines TNF- α and IFN- γ (Figure 3 and 4). The highest concentrations namely 2064.83 ± 172.79 and 96.03 ± 15.17 pg/mL, respectively, were elicited by an extract dose of 240 mg/kg bw. These levels were higher than those induced by the positive control, Stimuno[®] namely 1433.17 ± 419.83 and 79.34 ± 22.38 pg/mL.

Meanwhile, the dose extract of 60 mg/kg bw produced lower levels of TNF- α and IFN- γ , while the extract dose of 120 mg/kg bw also yielded a higher IFN- γ level than Stimuno[®]. The post hoc LSD analysis showed that the effect of the extract and the positive control Stimuno[®] differed significantly from the negative control ($p < 0.05$). This result indicates that the extract dose of 240 mg/kg bw can activate macrophage phagocytosis and significantly increase the level of TNF- α and IFN- γ .

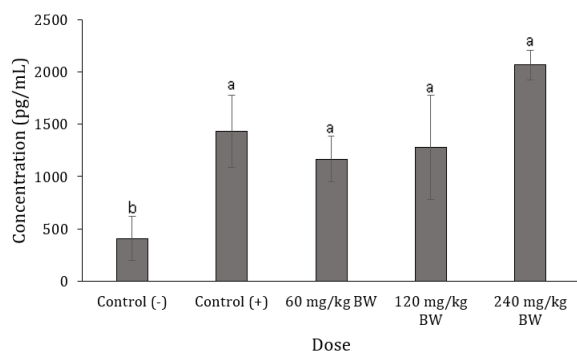


Figure 3. TNF- α level in rats after treated by *B. medicinalis* ethanol extract for 7 days. Data are presented as mean \pm SD of four observations. The means of the different letter superscript are significantly difference by post hoc LSD test at 0.05 significance level.

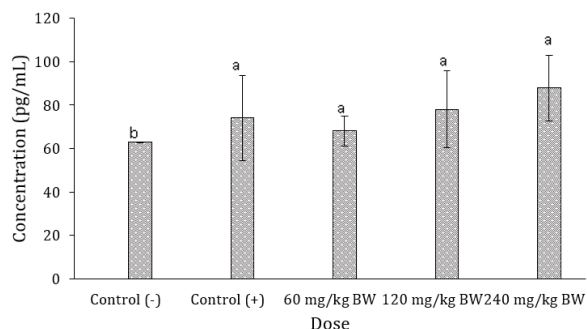


Figure 4. IFN- γ level in rats after treatment with *B. medicinalis* ethanol extract for 7 days. Data are presented as mean \pm SD of four observations. The means of the different letter superscript are significantly difference by post hoc LSD test at 0.05 significance level.

Based on the results, the immunostimulatory activity of *B. medicinalis* ethanol extract is a dose-dependent activator of macrophages to phagocytes. The significant

difference between each treatment group indicates that 240 mg/kg bw is the best dose. Therefore, further evaluation of cytokine production showed that there was a significant difference in the levels of TNF- α and IFN- γ between treatments of extract/Stimuno[®] and the negative control. The levels of TNF- α and IFN- γ in the treatment of 240 mg/kg bw were also higher than that of the positive control[®]. The high percentage of macrophage phagocytic activity was in line with the increasing levels of TNF- α and IFN- γ . TNF- α contributes directly to the acute immune response by affecting macrophage activation in the early stage, leading to the destruction, and elimination of pathogens. Meanwhile, IFN- γ mediates innate immunity in this acute-phase response (Hirayama *et al.*, 2017; Whiteside, 2007).

Total saponin, total phenolic and total flavonoid of *B. medicinalis* ethanol extract

Several studies reported that specific secondary metabolites mediate the immunostimulatory activity of the medicinal plant (Espírito-Santo *et al.*, 2017; Wang *et al.*, 2019). *B. medicinalis* reportedly contains saponin, flavonoids, and phenolics (Anam *et al.*, 2014; Khumaidi *et al.*, 2020; Zubair, *et al.*, 2021; Zubair *et al.*, 2020). The total saponin, phenolic, and flavonoid were determined using escin, gallic acid, and quercetin as standards with the equations of calibration curve $y = 0.00171x + 0.17155$ ($R^2 = 0.9828$), $y = 0.0156x + 0.0503$ ($R^2 = 0.9926$), and $y = 0.0142x + 0.0318$ ($R^2 = 0.9989$), respectively as shown in Figure 5 and Table 1. It was found that the extract contained more total saponin amounting to 319.72 ± 26.46 mg EE/g extract than phenolics and flavonoids of 112.02 ± 17.16 mg GAE/g and 11.35 ± 1.06 mg QE/g extract, respectively.

Flavonoids have been shown to increase IL-2 and lymphocyte proliferation which in turn, will affect CD⁴⁺ cells and activate Th1 cells. Furthermore, the activation of Th1 cells affects specific macrophage-activating factors. Flavonoids also activate NK cells to stimulate the production of TNF- α and IFN- γ (Brattig *et al.*, 1984; Martínez *et al.*, 2019). Phenolic compounds were also reported to activate β cells and increase the killing activity of NK cells (Kilani-Jaziri *et al.*, 2017). A saponin from green tea reportedly reduced the level of IL-10/IL-8 expression and increased TNF- α , IFN- γ , IL-1, IL-2, and IL-12 expression in T lymphocytes (Bhardwaj *et al.*, 2014).

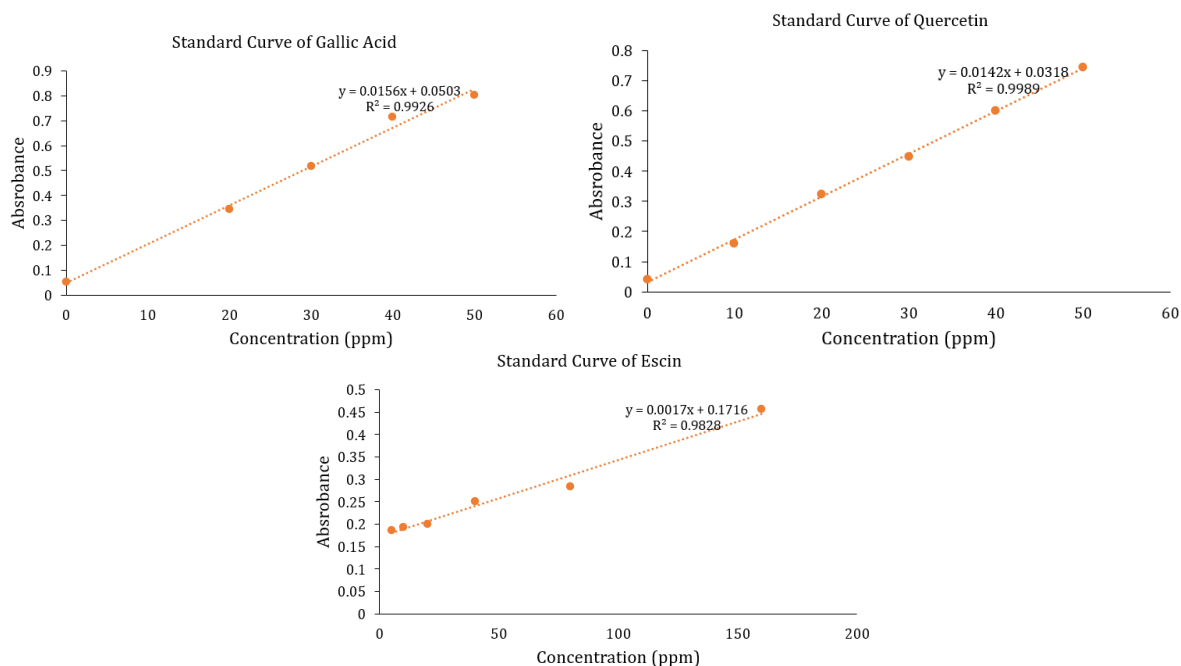


Figure 5. Standard curve of escin, gallic acid and quercetin

Table I. Total saponin, total phenolic and total flavonoid of *B. medicinalis* 70% ethanol extract.

Phytochemical analysis	Ethanol extract
Total saponin (mg EE/g extract ± SD)	319.72 ± 26.46
Total phenolic (mg GAE/g extract ± SD)	112.02 ± 17.16
Total flavonoid (mg QE/g extract ± SD)	11.35 ± 1.06

The high concentration of these metabolites in *B. medicinalis* extract might be responsible for the immunostimulatory activity, suggesting further investigation on the isolation of secondary metabolites and standardization of the extract as a raw material for immunomodulator herbal agents. This study also provides a scientific basis for the empirical application of this medicinal plant as an anticancer agent.

CONCLUSION

Ethanol extract of *B. medicinalis* activates macrophage phagocytosis in a dose-dependent manner. This activation was also proved by the significantly high production of cytokines TNF- α and IFN- α in rats along with increased concentrations of total saponin, phenolic, and flavonoid. Therefore, its development as a herbal remedy with immunostimulatory activity is proposed.

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