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Original Article

Immunomodulatory Effect of Methanolic Extract and Ethyl Acetate Fraction of Bengkoang (*Pachyrhizus erosus* (L.) Urban) Tuber in Mice

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ABSTRACT

Background: Recently, interest in the use of herbal medicine that can modulate the immune system is increasing in the pandemic situation. One plant that can be developed as an immunomodulator is bengkoang (*Pachyrhizus erosus* (L.) Urban). **Objectives:** To evaluate the immunomodulatory effect of a methanolic extract of bengkoang (MEB) and the ethyl acetate fraction of bengkoang (EAFB) in mice induced by the hepatitis B vaccine. **Materilas and Methods:** Thirty healthy male mice were divided into six groups namely, control, standard levamisole, MEB (100 and 200 mg/kg BW), and EAFB at 100 and 200 mg/kg body weight (BW). The treatments were given for 18 days, and hepatitis B vaccine was injected intraperitoneally twice during the treatment. Assessment of the immunomodulatory effect was carried out against nonspecific and specific immune response parameters. **Results:** The MEB and EAFB could significantly increase phagocytic capacity, the spleen index, and lymphocyte proliferation. MEB stimulated IgG production, while EAFB, 100 mg/kg BW suppressed immunoglobulin G (IgG) production; otherwise, at the higher dose, EAFB increased IgG production. EAFB also increases nitric oxide production, while MEB had no effect. The higher dose of MEB tended to increase tumor necrosis factor (TNF)-α levels and decrease interleukin (IL)-10, while EAFB tended to decrease TNF-α and increase IL-10, but these changes were not significant. **Conclusion:** Based on this study, MEB and EAFB could increase the innate immune response and stimulate the humoral immune response but had no effect on cytokine production, which may have potential usefulness of bengkoang to treat immunomodulatory-related disease.

Key words: Bengkoang (*Pachyrizus erosus* (L.) Urban), Dietary fiber fraction, Immunomodulatory effect, Phagocytic macrophages activity, Lymphocyte proliferation.

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INTRODUCTION

Currently, public interest in the use of herbal medicine as an agent that can modulate the immune system is increasing. Immunomodulators are compounds that can modulate the immune system by stimulation or suppression.[1] Chemical compounds in some plants, such as polysaccharides, flavonoids, diterpenoids, alkaloids, and glycosides, have been reported to be responsible for immunomodulatory effects.[2] One plant that can be developed as an immunomodulator is bengkoang (*Pachyrhizus erosus* (L.) Urban), an edible root vegetable that contains micro- and macronutrients, large amounts of ascorbic acid, fiber, starch, and water. Bengkoang also contains inulin, and a rather low caloric content (39 kcal/100 g), has benefits to human health, and is potentially used as a functional food.[3] Bengkoang also contains chemical compounds such as saponins (terpenoid glycosides), namely kaikasaponin III and phaseoside IV.[4] Lukitaningsih and Holzgrabe reported four compounds isolated from EAFB, three of them isoflavonoids in the form of aglycon and glycosides, namely daidzein, daidzein-7-O-ß-glucopyranose, and 5-hydroxy-daidzein-7-O-*β* glucopyranose. One other compound, 8,9 furanyl-pterocarpan-3-ol, is the pterocarpan group.[5]

Previous research reported that the isoflavone daidzein has bioactivities such as anti-inflammatory and immunomodulatory effects. Saponin also has biological activities such as an immunomodulation.[6] Nowadays, several studies of the immunomodulatory effect of bengkoang have been reported. Kumalasari et al. published that bengkoang fiber extract (BFE) stimulated phagocytic activity in vitro (J774.1 cells) and in vivo. BFE also facilitated the production of IL-6 and TNF-α by J774.1 cells and mouse peritoneal macrophages.[7] BFE facilitated the production of IgM, IgG, and IgA and the cytokines IL-5 and IL-10 in mouse splenocytes after oral administration of BFE for 2 weeks.[8] The BFE-B fraction of a water-soluble fiber was reported to have an immunomodulatory effect on the innate and adaptive immune responses.[9] The above study indicates that bengkoang tuber has the potential for development as a functional food that is efficacious as an immunomodulator.

However, immunomodulatory research about bengkoang is still limited to bengkoang fiber extract (BFE); there have been no studies about the immunomodulatory effects of methanolic extract (MEB) containing polar chemical compounds such as saponin and ethyl acetate fractions of bengkoang (EAFB) containing isoflavone daidzein, which were thought to have an immunomodulatory

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effect. Therefore, this study was performed to investigate the immunomodulatory effect of MEB and EAFB against innate and adaptive immune responses in Balb/c mice induced by hepatitis B vaccine.

MATERIALS AND METHODS

Chemicals

Methanol, petroleum ether, ethyl acetate, NH_4Cl, $NaHCO_3$, HCl, $NaNO_2$, sodium carboxymethyl cellulose (CMC-Na), sulfanilamide, phosphoric acid, N-(1-naphthyl) ethylenediamine dihydrochloride, tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)), phytohemagglutinin, penicillin-streptomycin, Giemsa, and latex beads were purchased from Sigma–Aldrich Pte. Ltd., Singapore. Rosewell Park Memorial Institute (RPMI) 1640, phosphate-buffered saline (PBS), fetal bovine serum (FBS), and fungizone were purchased from GIBCO, USA. Levamisole was obtained from PT Konimex, Indonesia. Hepatitis B vaccine (Engerix-B) was obtained from Glaxo Smith Kline. Mouse IgG, TNF-α, and IL-10 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Fine test, Wuhan Fine Biotech Co. Ltd., China.

Plant material collection

Pachyrhizus erosus tubers were harvested from Prembun Kebumen, Central Java of Indonesia. Plants species was identified by Dr. Djoko Santoso from the laboratory of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia with number: UGM/FA/2254/M/03/02.

Animals

Healthy male BALB/c mice, 8–10-weeks old and 20–30 g, were obtained from the Laboratory of Pharmacology and Toxicology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. The animals were housed and maintained under standard conditions with a 12-h light/dark cycle, a temperature of 25 ± 2°C, and humidity of 60%–70%. Animals were acclimatized 1 week before experimentation and randomized into treatment and control groups. All procedures associated with animal experiments were carried out following the guidelines for the care and use of laboratory animals. All experimental procedures were approved by The Animal Ethics Committee of the Integrated Research and Testing Laboratory, Universitas Gadjah Mada (No. 00073/04/LPPT/VII/2018).

Extraction and fractionation of bengkoang

Fifty kilograms of fresh bengkoang tubers were cleaned, chopped, then dried at 40–50°C in the oven for 2 days. The dried tuber was then powdered by blending. Bengkoang powder (4.3 kg) was extracted using petroleum ether to obtain nonpolar compounds. The residue was extracted using methanol. The concentrated methanol extract was combined with water and then partitioned with ethyl acetate. The ethyl acetate phase was collected and then concentrated.[5]

Animal experimental

An experimental study was performed with a post-test only design including a control group. Thirty mice were divided randomly into six groups of five mice. Group I was given 0.5% sodium carboxymethyl cellulose (CMC-Na), group II was treated with levamisole 2.5 mg/kg BW, and groups III–VI were administered MEB (100 and 200 mg/kg BW) and EAFB (100 and 200 mg/kg BW), respectively. Treatment was given orally once daily for 18 consecutive days. During the treatment, on days 7 and 14, all mice were injected intraperitoneally with hepatitis B vaccine 2.6 µL/20 g BW.[10] On days 19, mice were anesthetized with ketamine 180 mg/kg BW before blood sampling from the retro-orbital plexus. The serum was used for the determination of IgG, TNF-α, and IL-10 with an ELISA kit following the manufacturer's protocol. Then, the mice were sacrificed, peritoneal fluid was taken for phagocytosis and the nitric oxide assay, and the spleen was removed for determination of the spleen index and the lymphocyte proliferation test.

Isolation of macrophage and phagocytosis activity assay

After mice were sacrificed, the skin of the abdomen was opened aseptically. Macrophages were isolated from peritoneal fluid by injecting 10 mL of cold RPMI, then centrifuged at 2000 rpm and 4°C for 10 min. The supernatant was removed, and pellets were suspended in complete RPMI medium to a density of 2.5 x 10^6 cells/mL, then cultured on 24-well microplates, which were covered by coverslips and incubated overnight at 37°C in a 5% CO_2 incubator. Further, the medium was removed and washed once with RPMI. Subsequently, 1 mL of complete RPMI medium was added to each well and incubated for a further 4 h. A 300-µL suspension of latex beads with a density 2.5 × 10^7 beads/mL was added to each well, then incubated at 37°C in a 5% CO_2 incubator for 60 min. The cells were then washed with PBS 3 times, then dried at room temperature. The cells were fixed with methanol for 30 s, then the methanol was removed. Afterward, the coverslips were stained with Giemsa 20% v/v for 30 min, then washed with distilled water and dried at room temperature. The macrophage phagocytosed latex beads were counted using a light microscope at 400× magnification. The activity of macrophage phagocytosis was evaluated using the phagocytic index and phagocytotic capacity.[11]

$$\%\ phagocytotic\ capacity = \frac{\Sigma\ macrophages\ that\ phagocytosed\ latex\ beads}{100\ macrophages\ counted} \times 100$$

$$Phagocytic\ index = \frac{\Sigma\ latex\ beads\ that\ were\ phagocytosed\ by\ 100\ macrophages}{\Sigma\ active\ macrophages}$$

Nitric oxide (NO) production in peritoneal macrophages

A 2000 µM stock solution of $NaNO_2$ was made by weighing 69 mg of $NaNO_2$, then dissolved in 100 mL double distilled water. $NaNO_2$ solution from 0 to 100 µM was used as a standard. Peritoneal cells with a density of 1 x 10^6 cells/mL were cultured in 24-well plates and incubated for 24 h at 37°C in an incubator with 5% CO_2. The sample (supernatant) was removed, and a series of concentrations of $NaNO_2$ from 0 to 100 µM was incorporated into 96-well microplates in triplicates of 100 µL each, 100 µL Griess reagent.[12] Then a mixture of Griess A and B (1:1), was added to each well and incubated for 15 min at room temperature, and the absorbance was measured at 550 nm using an ELISA microplate reader.

Spleen index determination

One day after the last treatment, mice were sacrificed, and the spleen was removed and weighed. The spleen index was calculated according to the following formula:

$$Spleen\ index\ (\%) = \frac{Spleen\ weight\ (mg)}{body\ weight\ of\ mouse\ (g)} \times 100$$

Lymphocyte isolation and proliferation assay

The spleens of mice were isolated aseptically, 10 mL of RPMI was pumped into the spleen until the lymphocyte cells suspension was completely transferred to a 50-mm petri dish, then the cell suspension was centrifuged at 2000 rpm, 4°C for 10 min. The supernatant was decanted, and the pellet was suspended in Tris-buffered NH_4Cl to lyse erythrocytes and left at room temperature for 5 min. Centrifugation was repeated for 10 min, and the supernatant was discarded. Pellets

were suspended in complete RPMI medium to a density of 1.5 x 10^6 cells/mL, then cultured at 100 µL/well in 96-well microplates. Phytohemagglutinin (2 µL/well) was added to each well and incubated in a 5% CO_2 incubator at 37°C for 48 h. Furthermore, 10 uL MTT 5 mg/mL solution was added to each well, then incubated again under the same conditions for 4 h. Then, 100 µL stop reagent was added to each well. Lymphocyte cell proliferation was measured from the optical density with an ELISA microplate reader at 550 nm.

Production of antibody (IgG) and cytokines TNF-α and IL-10

Blood was taken from the retro-orbital plexus on the 19th day. Blood samples were allowed to stand for 1 h at room temperature. Blood was centrifuged at 4000 rpm for 10 min, and the supernatant (serum) was stored at −80°C until use. The serum was used for the determination of IgG, TNF-α, and IL-10 production using mouse ELISA kits.

Statistical analysis

All data are presented as mean ± SEM (standard error of the mean). Statistical analysis of the experimental results was performed by one-way ANOVA followed by the Least Significant Difference post hoc test (2-tailed) or the Kruskal–Wallis followed by the Mann–Whitney U test, with a confidence level of 95% using SPSS version 22. A (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ were considered statistically significant.

RESULTS

Phagocytic activity in peritoneal macrophages

The administration of MEB and EAFB 100 and 200 mg/kg BW could enhance the capacity of phagocytic activity by a factor of approximately 2.5 compared with the negative control ($P < 0.05$), as shown in Figure 1A. The MEB dose of 100 mg/kg BW increased the phagocytic index significantly to 3.47 (Figure 1B), while EAFB did not influence the phagocytic index.

Production of nitric oxide (NO) in peritoneal macrophages

The result demonstrated that NO production in groups given levamisole and EAFB was significantly higher than that in the control ($P < 0.05$) (Table 1). Meanwhile, the groups treated with MEB 100 and 200 mg/kg BW showed no difference relative to the control group. This shows that the potential for NO production in animals treated with MEB and EAFB differs.

Spleen index

The spleen is one of the secondary lymphoid organs; these organs contain T and B lymphocyte cells, which function in the process of specific immunity. Table 2 shows that in all treatment groups (levamisole, MEB, and EAFB), the spleen index increased significantly ($P < 0.05$). Administration with EAFB 200 mg/kg BW presented the highest spleen index. These results represent that the immune responses of mice induced by hepatitis vaccine were enhanced following 18 days of MEB and EAFB administration.

Lymphocyte proliferation assay

Figure 2A shows increasing the optical density of lymphocyte cell proliferation in the groups treated with levamisole, MEB, and EAFB compared with the negative control ($P<0.05$). This result indicates that MEB and EAFB could stimulate lymphocyte cell proliferation or could modulate one of the specific immune responses. In this study, the addition of phytohemagglutinin as a mitogen selectively stimulated T-lymphocytes.[13]

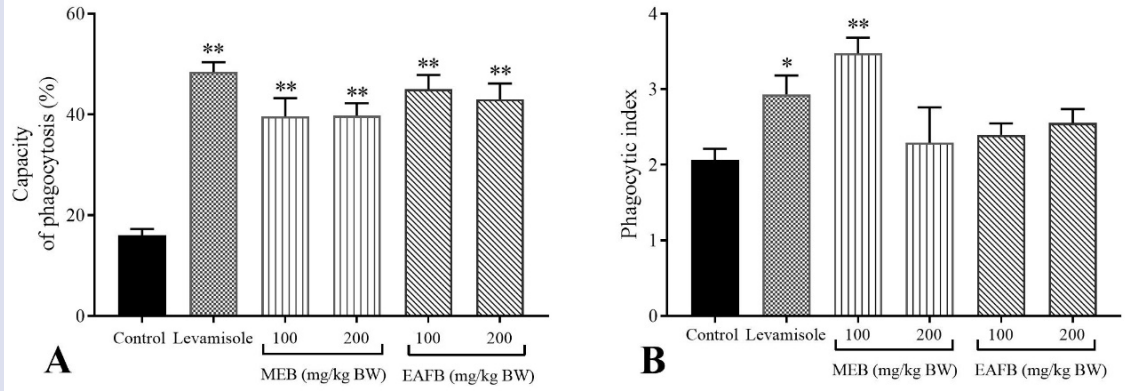

Figure 1: Effect of a methanolic extract (MEB) and the ethyl acetate fraction of bengkoang (EAFB) on the capacity for phagocytosis (A) and the phagocytic index (B) of peritoneal macrophages (mean ± SEM, n = 5). *$P < 0.05$, **$P < 0.01$ was significantly different compared with the control.

Table 1: Effect of a methanolic extract of bengkoang (MEB) and the ethyl acetate fraction of bengkoang (EAFB) on nitric oxide production in peritoneal macrophages.

Group	Nitric oxide concentration (µM)
Control (0.5% CMC-Na)	0.743 ± 0.188
Levamisole 2.5 mg/kg BW	2.731 ± 0.343**
MEB, 100 mg/kg BW	1.341 ± 0.246
MEB, 200 mg/kg BW	1.072 ± 0.199
EAFB, 100 mg/kg BW	3.177 ± 0.916**
EAFB, 200 mg/kg BW	2.896 ± 0.576**

Antibody (immunoglobulin G) and cytokine production

The results demonstrated that IgG production was stimulated by MEB at doses of 100 and 200 mg/kg BW and EAFB at 200 mg/kg BW compared with the control ($P < 0.05$). Meanwhile, IgG production was significantly suppressed with a low dose of EAFB (Figure 2B). The administration of MEB with a dose of 200 mg/kg BW increased TNF-α compared with the control ($P < 0.05$); otherwise, EAFB at that dose did not differ from the control (Figure 3A). Meanwhile, IL-10 production increased slightly with administration of MEB and EAFB, although there were no significant differences from the control (Figure 3B).

DISCUSSION

The immunomodulatory effect of MEB and EAFB were evaluated using non-specific and specific immune response parameters. Macrophages play an important role in representing a non-specific first-line defense against invading antigens such as viruses. In this study, the activation of macrophages was determined based on the ability of macrophages to phagocytose latex beads and to produce nitric oxide. Specific immune responses were evaluated according to the parameters lymphocyte proliferation, spleen index, and immunoglobulin production. Lymphocyte cells function to maintain specific immune responses

Table 2: Effect of a methanolic extract of bengkoang (MEB) and the ethyl acetate fraction of bengkoang (EAFB) on the spleen index.

Groups	Spleen index (%)
Control (0.5% CMC-Na)	0.459 ± 0.028
Levamisole 2.5 mg/kgBW	0.694 ± 0.013**
MEB, 100 mg/kg BW	0.686 ± 0.048**
MEB, 200 mg/kg BW	0.734 ± 0.061**
EAFB,100 mg/kg BW	0.641 ± 0.020*
EAFB, 200 mg/kg BW	0.829 ± 0.043**

The data represent the mean ± SEM (n = 5). *$P < 0.05$, **$P < 0.01$ was significantly different compared with the control.

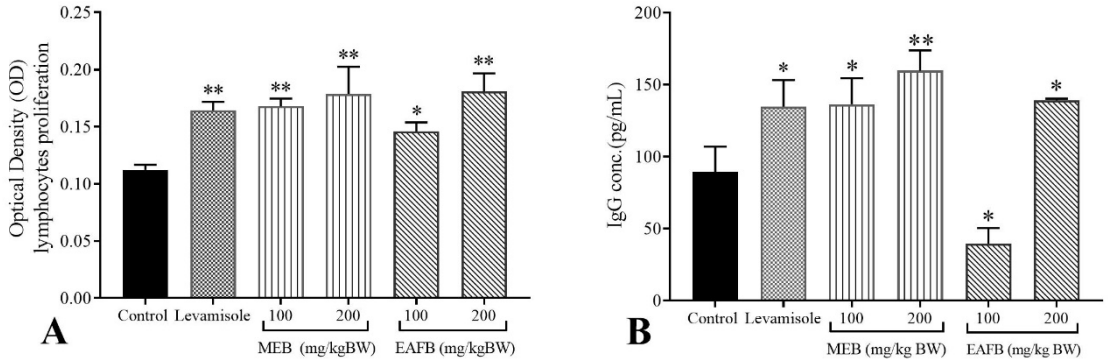

Figure 2: Optical density of lymphocyte proliferation (A) and the concentration of IgG (B) after treatment with methanolic extract of bengkoang (MEB), ethyl acetate fraction of bengkoang (EAFB) (mean ± SEM, n = 5). *$P < 0.05$, **$P < 0.01$ was significantly different from the control.

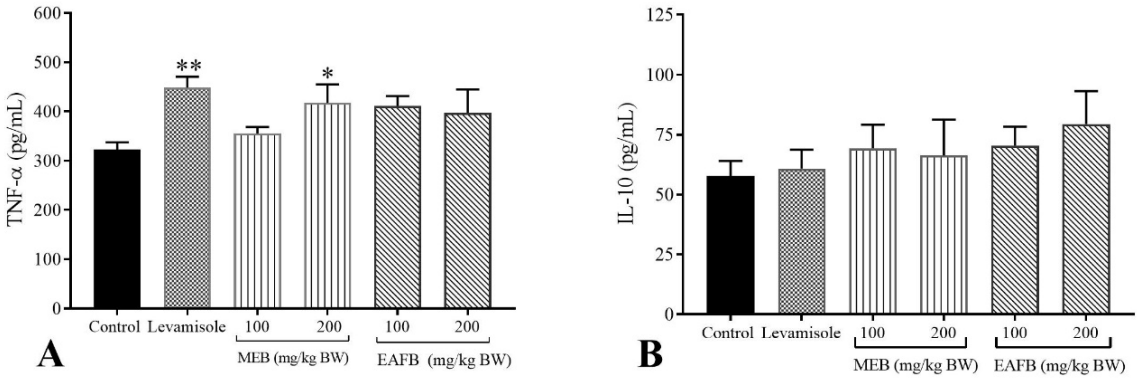

Figure 3: Concentration of TNF-α (A) and IL-10 (B) in mice after administration of methanolic extract of bengkoang (MEB) and ethyl acetate fraction of bengkoang (EAFB) (mean ± SEM, n = 5). *$P < 0.05$, **$P < 0.01$ was significantly different compared with the control.

including cellular (lymphocytes related to T cells) and humoral (involving antibodies) immune responses.[1]

The entry of foreign matter, such as hepatitis B vaccine, into the body will stimulate B lymphocytes to differentiate, proliferate, and develop into plasma cells that produce antibodies (immunoglobulins). Measurement of IgG levels was carried out on the 19th day because IgG reaches its peak between the 10th and 14th days. When there is repeated antigen entry, a lag phase appears usually between days 3 and 5 after a repeat infection, and IgG levels increase much higher, and the increase lasts longer.[1] Cytokines are glycoproteins derived from T helper (Th) cells, natural killer (NK) cells, and macrophages, which play an important role in the immune response against pathogens. Th cells consist of two subsets, Th1 and Th2, that produce cytokines. Th-1 produce cytokines that will activate macrophages to form proinflammatory cytokines such as TNF-α and activate macrophages to induce cytotoxic effector immune mechanisms. Instead, Th-2 produce cytokines such as IL-10 to induce antibody formation but inhibit macrophage function (called anti-inflammatory cytokines).[1] The previous study reported that hepatitis B vaccine can increase the production TNF-α.[14]

Nitric oxide can be produced during the activation of macrophages by antigens such as viruses. Nitric oxide is toxic to pathogenic bacteria. This result demonstrated that the capacity and phagocytic index of MEB was relatively high, but nitric oxide was not produced in excess. This effect was possible because the chemical compound in the sample could maintain immune function by protecting against excessive macrophage phagocytosis activity.[15]

Based on this study, MEB showed an immunostimulatory effect on the innate immune response by significantly enhancing both the macrophage phagocytotic capacity and the phagocytic index. MEB also could modulate the adaptive immune response by increasing lymphocyte proliferation and the spleen index and stimulating IgG production. This activity was possible because MEB contains polar chemical compounds such as saponin.[4] Naknukool et al. reported that saponins can enhance the immune response through macrophage stimulation.[6] Iqbal et al. reported that saponins can stimulate lymphocyte proliferation.[16] Previous research indicated that saponin as an adjuvant can modulate the cellular immune system and increase antibody production.[17]

EAFB also presented an immunostimulatory effect on the innate immune response by increasing the capacity of phagocytosis and nitric oxide production. EAFB affected the adaptive immune response by increasing the spleen index and lymphocyte proliferation and stimulating IgG production at the higher dose (200 mg/kg BW), while at a low dose of EAFB (100 mg/kg BW) significantly suppressed IgG production. This immunomodulatory effect was a possibly caused by semipolar compounds contained in EAFB, such as isoflavone daidzein.[5] These effects were in line with other research. Maji et al. reported that daidzein exerts immunomodulatory effects by increasing the phagocytic index; the total number of leukocytes, monocytes, and lymphocytes increased in a dose-dependent manner.[18] Daidzein stimulated IgM and IgG titers expressed in the form of hemagglutination antibody titers.[19] Other studies reported that daidzein supplementation increases IgG levels[20,21]; IgG increased significantly in mice fed a diet high in soy (containing daidzein).[22] Saybel et al. reported that phenolic compounds such as flavonoids can affect cell-mediated and humoral immunity as well as macrophage phagocytosis.[23]

Levamisole (imidazothiazole group) is a synthetic drug that induces B and T lymphocytes, monocytes, and macrophages. In this study, levamisole as a reference drug could increase phagocytosis of macrophages, NO production, the spleen index, and lymphocyte proliferation and stimulate IgG and TNF-α production. These results were in line with several studies that reported levamisole could increase phagocytosis of macrophages and the levels of IFN-γ, IL-5, TNF-α, IgM, and IgG.[24-25]

This study showed that a higher dose of MEB tended to increase levels of the cytokine TNF-α, while IL-10 production decreased, although the changes were not significant. The higher dose of EAFB decreased TNF-α production (inflammatory cytokines), whereas IL-10 (anti-inflammatory cytokine) production slightly increased, but the changes were not significant. These indicate that the higher dose of EAFB increases the anti-inflammatory effect (although the difference was not significant). Other research demonstrated that the isoflavone daidzein has potential as an anti-inflammatory.[26] These results show that EAFB has an immunomodulatory effect, but suggest the need for further examination using higher doses to determine whether at the higher dose EAFB has an anti-inflammatory effect, and it is necessary to determine the concentration of saponins and isoflavone daidzein contained in MEB and EAFB.

CONCLUSION

It can be concluded that the methanolic extract of bengkoang (MEB) and ethyl acetate fraction of bengkoang (EAFB) have an immunomodulatory effect via enhancement of the nonspecific immune response. MEB and EAFB at a dose of 200 mg/kg BW also stimulated IgG production (humoral immune response) but had no influence on production of the cytokines IL-10 and TNF-α. These findings suggest that bengkoang might have a potentially beneficial effect on immune system-related diseases.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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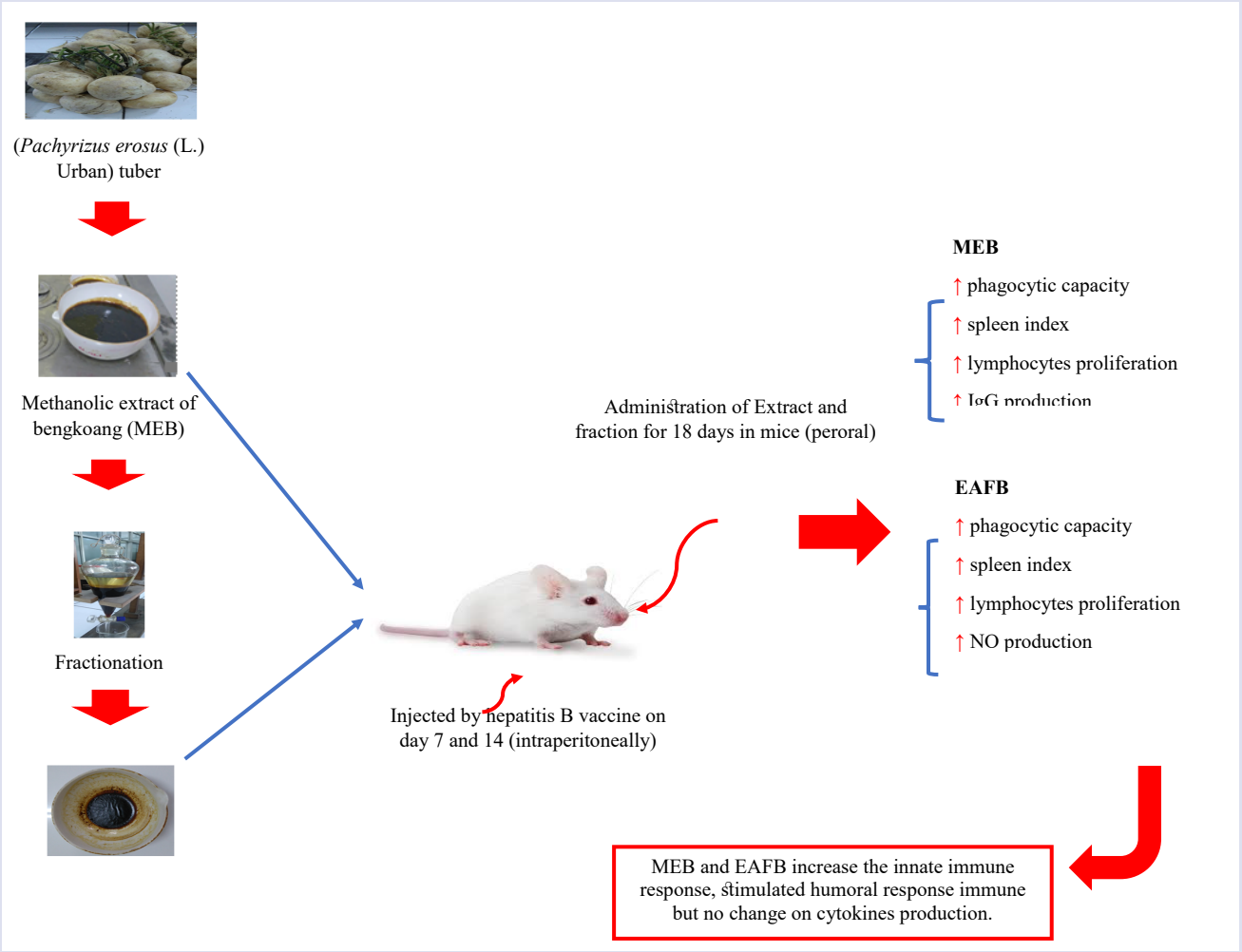
GRAPHICAL ABSTRACT



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