



Immunomodulatory effects of standardized extract of *Curcuma mangga* val. on cytokines, antibody and delayed-type hypersensitivity response in Wistar rats

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Abstract

Background and purpose: Recently, we have highlighted the immunomodulatory activity of *Curcuma mangga* Val. on phagocytosis ability. The current study was conducted to determine the immunomodulatory effects of the standardized extract of *C. mangga* rhizomes by *in vitro* and *in vivo* studies.

Experimental approach: The *C. mangga* extract was standardized according to a guideline for herbal preparation. The extract was investigated for its immunomodulatory effects on gene expression of cytokines, cytokines and antibody production as well as delayed-type hypersensitivity (DTH) response. The gene expression of cytokines on lipopolysaccharide-induced-RAW 264.7 cells was analysed by reverse transcription-polymerase chain reaction (RT-PCR) method. The effect of the extract on DTH response was investigated by the paw edema method, meanwhile the effects of the extract on antibody and cytokine production from normal and cyclophosphamide-induced *Salmonella typhimurium* infected rats were determined using an enzyme-linked immunosorbent assay (ELISA).

Findings/Results: The extract of *C. mangga* demonstrated an inhibitory effect on gene expression of interleukin-1 β (IL-1 β), tumor necrosis factor- α , and IL-6 as compared to lipopolysaccharide-induced cells. The extract also depicted inhibitory activity on IL-4 production as compared to the negative control. Whereas, the DTH response and production of immunoglobulin G from both groups after treatment with *C. mangga* extract were higher than those of negative control ($P < 0.05$).

Conclusion and implications: The results indicated that the *C. mangga* extract has immunomodulatory effects, emphasizing its potential to be developed as immunotherapeutic agent.

Keywords: Standardized extract of *Curcuma mangga*; immunomodulatory; delayed-type hypersensitivity; immunoglobulin; cytokines.

INTRODUCTION

The human body has developed a remarkably sophisticated defense system (1). This system is mediated by various cells and molecules which are able to recognize and destroy the various pathogen and undesirable materials (2). Phagocytes play an important role in innate immunity, meanwhile adaptive immunity is mainly facilitated by lymphocytes. Lymphocytes T cells are subdivided into two distinct lineages that can be differentiated

by their expression of CD4⁺ or CD8⁺ markers on their surface. Activation of lymphocytes CD4⁺ T cell initiates delayed-type hypersensitivity (DTH) response (3).

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Several molecules are involved in the host defense, which includes antibodies (immunoglobulins), cytokines, and complement. Cytokines are a large group of molecules which involve in the signaling process between cells during immune responses (4). Cytokines perform their function through the interaction with cytokine receptors that can be grouped in several distinct families (5). Initially, cytokines were believed to act mainly as antiviral or antineoplastic agents. Nowadays, they are known to be crucial to innate and adaptive immunity, cell growth, and differentiation (6). They can be produced by T cells, mononuclear phagocytes, or by tissue cells. Antibodies are also humoral components which play a necessary role in adaptive immunity (4). Defects or malfunctions in the immune system can cause pathological conditions. Some drugs have been reported to impair host defense, such as cyclophosphamide, cyclosporine, and dexamethasone (7). Modulation of immune response by an immunomodulator is important to treat those diseases due to malfunction in the immune system. Natural medicines are the main sources of safe and effective immunomodulators.

Since ancient times, the focus on the use of traditional medicines has gained momentum all over the world. Many medicinal plants such as *Brassica rapa* L., *Curcuma mangga*, and *Cucurbita pepo* have been used in folk medicine (8,9,10). *Curcuma mangga* rhizome has been used in the treatment of cancer, fever, and stomach disorders (9). It was found to have a variety of active secondary metabolites, such as flavonoids, saponins, glycosides, terpenoids, and steroids (11). Our previous study has displayed the immunomodulatory activities of *C. mangga* on phagocytosis, as well as delayed type hypersensitivity reaction and titer antibody by *in vivo* study in bovine-red blood cells challenged-rats (11,12). *C. mangga* has also been evaluated for its toxic effect which revealed that its LD₅₀ was more than 5000 mg/kg BW, indicating that it was nontoxic (13). The *in vitro* nitric oxide inhibitory activity of methanol extract of *C. mangga* has also been reported (14). *C. mangga* has also been found to have analgesic and anti-inflammatory

activities (15,16). However, the effects of *C. mangga* on DTH response of *Salmonella typhimurium*-infected rats and inflammatory cytokines at gene level as well as cytokine and immunoglobulin G production have not been reported. The current study was performed to evaluate the immunomodulatory effects of ethanol extract of *C. mangga in vitro* using RAW 264.7 macrophages and *in vivo* using normal and cyclophosphamide-induced *S. typhimurium*-infected rats. *in vitro* study was conducted to evaluate the effect of *C. mangga* extract on cytokine gene expression, meanwhile *in vivo* study was performed to investigate the effect of the extract on cytokine and antibody production as well as DTH response in Wistar rats.

MATERIALS AND METHODS

Chemicals and reagents

The chemicals used in this study were n-hexane, ethyl acetate, and ethanol which were purchased from Smart Lab (Indonesia). Lipopolysaccharide (LPS) from *Escherichia coli* O111.B4 (Sigma, USA) and Griess reagent (Sigma, USA) were used in this study. Phosphate buffer saline (PBS; Sigma, USA), sodium carboxymethyl cellulose (Na CMC; Sigma, USA) and levamisole (Askamex[®]; Soho, Indonesia), Dulbecco's modified eagle medium (DMEM; Biowest, USA), fetal bovine serum (FBS; Gibco, USA), penicillin-streptomycin (Gibco, USA), Fungizone[®] (amphotericin B; Sigma, USA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma, USA), 3-(4,5-dimethyliazol-2-yl)-2,5-difeniltetrazolium bromide (MTT; Sigma, USA), PBS (Irvine Scientific, USA) were also used in this study. Total RNA mini kit (Geneaid, Taiwan), ReverTra Ace (Toyobo, Japan), GoTaq[®]Green (Promega, USA), nuclease-free water (Promega, USA), tris-borate-EDTA (Vivantis, Malaysia), agarose gel (Promega, USA), FluoroVue (Smobio, Taiwan), DNA ladder 100 bp (Smobio, Taiwan) were used to evaluate the gene expression. Microplate reader (Bio Rad, USA), electrophoresis apparatus (BioRad, USA) and polymerase chain reaction (PCR; ProFlex, UK) were also used in this study.

The FineTest® ELISA kits were obtained from Wuhan Fine Biotech Co., Ltd. (China).

Plant materials

The plant collection was performed in North Sumatera, Indonesia. The plant sample was identified by a biologist in Herbarium Medanese (MEDA), the University of Sumatera Utara, Indonesia (Voucher No. 4536/MEDA/2019).

Extraction procedure

The rhizomes were dried, ground, then extracted by maceration method using ethanol as the solvent. Briefly, the dried plant material (500 g of *C. mangga* rhizomes) was macerated with ethanol (1:10). Then, the solvent was removed using a rotary evaporator to yield ethanol extract of *C. mangga* rhizomes (11).

Standardization of *C. mangga* extract

The procedure of standardization of *C. mangga* extract is divided into 2 categories, namely specific and nonspecific standardization according to the guidelines released by The National of Drug and Food Control, Indonesia (17).

Cell culture

RAW 264.7 macrophage cells were cultured in DMEM which supplemented with 10% FBS, penicillin and streptomycin. Then, cells were incubated in 5 % CO₂ at 37 °C (18).

Antigen preparation

S. typhimurium was cultivated in nutrient broth and incubated for 24 h. Aliquot of 1 mL was added with 9 mL nutrient broth, then the cell concentration was calculated using the spectrophotometry method until the

concentration of 1×10^8 cells/mL was obtained. Furthermore, it was centrifuged at 10,000 rpm for 10 min at 25 °C. The supernatant was discarded. Then, the cells were resuspended with 1 mL of PBS.

Cell viability assay

The cell viability evaluation was examined by MTT assay. RAW 264.7 macrophages were incubated for 24 h with LPS (1 µg/mL) and the extract (12.5-50 µg/mL). The viable cells were counted by measuring the absorbance using a microplate reader at 595 nm (19).

Cytokine gene expression assay

The gene expression of interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α) was determined using RT-PCR according to a previous study (20). Briefly, RAW 264.7 cells (5×10^5 cells/well) were incubated for 24 h in a 5% CO₂ incubator at 37 °C. Then, induced by LPS (1 µg/mL) after medium replacement, followed by incubation for 6 h, then incubated again with extract (25 µg/mL) for another 24 h. The total RNA mini kit (Geneaid, Taiwan) was used to extract total RNA from normal control (RAW 264.7 cells only) and negative control (LPS) cells and treatment groups (*C. mangga* extract). The oligonucleotide primers were referred to in a previous study (Table 1). PCR was performed with 35 cycles, including 1 min at annealing temperature (55 °C for TNF-α, IL-6, and β-actin, 62.5 °C for IL-1β) and 45 s at 95 °C for denaturation and 1 min at 72 °C for elongation. The β-actin was used as a housekeeping gene. PCR products were separated electrophoretically using agarose, thereafter the stained gel was visualized using Gel-Doc.

Table 1. Oligonucleotide primers for Cytokines and β-actin

Genes		Primer sequence	Size (bp)	Annealing temperature (°C)
Interleukin -1β	Forward	5'-CCCTGCAGCTGGAGAGTGTGGA-3'	447	62.5
	Reverse	5'-TGTGCTCTGCTTGTGAGGTGCTG-3'		
Tumor necrosis factor-α	Forward	5'-TGTGCCCGCTGTCTGCTTCACGCT-3'	374	55
	Reverse	5'-GATGAGGAAAGACACCTGGCTGTAGA-3'		
Interleukin -6	Forward	5'-GATGCTACCAAACCTGGATATAATC-3'	269	55
	Reverse	5'-GGTCCTTAGCCACTCCTTCTGTG-3'		
β-actin	Forward	5'-TGGAATCCTGTGGCATCCATGAAAC-3'	349	55
	Reverse	5'-TAAAACGCAGCTCAGTAACAGTCCG-3'		

Animals

Male Wistar rats weighing 120-200 g were used. The procedure was evaluated by Animal Research Ethics Committees (AREC), University of Sumatera Utara (Ethic No. 0560/KEPH-FMIPA/2019).

DTH response

The DTH response evaluation was conducted by measuring paw volume according to a modified previous method (21). The study was performed in normal and cyclophosphamide-induced rats. Animals were divided into 5 groups for each condition, including Na CMC 0.5% suspension, *C. mangga* extract 100 mg/kg BW, *C. mangga* extract 200 mg/kg BW, *C. mangga* extract 400 mg/kg BW, levamisole 25 mg/kg BW. *C. mangga* extract suspension in Na CMC 0.5% was orally administered to animals 72 h prior to sensitization with *S. typhi* (1×10^8 cells/mL) by intraperitoneal injection and continued until 14 days. Meanwhile, the negative control group received vehicle only (Na CMC 0.5%). Cyclophosphamide (30 mg/kg BW) was used to suppress the immune response as reported in a previous study (22). Cyclophosphamide was administered on days 4, 8, and 12 in immune-suppressed groups. Levamisole (25 mg/kg BW) was used as a positive control. On day 14, the paw volume was measured, then, all the rats were challenged by the same amount of *S. typhimurium* in the hind footpad. The DTH response was measured 24 h later and expressed by the mean increase of paw volume.

Immunomodulatory assay on antibody and cytokine production

The effect of the extract on immunoglobulin G (IgG) and cytokine (IL-4) production was evaluated following a modified previous method by Ahirwal *et al.* (21). The animals were treated with ethanol extract of *C. mangga* at 100, 200, and 400 mg/kg BW 72 h prior to sensitization with *S. typhimurium* (1×10^8 cells/mL) by intraperitoneal injection and continued until 14 days. Meanwhile, the negative control group received Na CMC 0.5% as the vehicle. Cyclophosphamide (30 mg/kg BW) was administered on days 4, 8, and 12 in immune-suppressed groups. On day 14, all the animals

were challenged by injection of the same amount of *S. typhimurium*. On day 15, the blood was taken and the serum was used for the determination of IgG and IL-4. Levamisole was used as a positive control at 25 mg/kg BW. The appropriate ELISA kits were used to measure the cytokine and antibody levels.

Statistical analysis

The data were presented as mean \pm SD and analyzed with SPSS software using a one-way analysis of variance (ANOVA) and followed by the post hoc Tukey test.

RESULTS

Standardization of *C. mangga* extract

The result of specific standardization of *C. mangga* extract consists of organoleptic parameters as shown in Table 2. The result of nonspecific parameters which include water content, total ash content, acid insoluble ash particles, Pb contamination, Cd contamination, total plate count, and yeast count met the requirement of The National of Drug and Food Control, Indonesia (Table 3). The National of Drug and Food Control required-water content level on standardized extract must be less than 10%. The extract with a water content that exceeds 10% can be easily contaminated by yeast and molds.

Table 2. Organoleptic parameters of standardized *Curcuma mangga* extract.

No	Parameters	Result
1	Color	Brown
2	Smell	Distinctive Smell
3	Form	Thick
4	Taste	Bitter Taste

Table 3. Non-specific parameters of standardized *Curcuma mangga* extract.

No	Parameters	Results
1	Water content	3.71 %
2	Total ash content	3.71 %
3	Acid insoluble ash particles	1.44 %
4	Pb contamination	0 ppm
5	Cd contamination	0 ppm
6	Total plate count	0.595×10^{-4} (CFU/g)
7	Yeast count	0.745×10^{-4} (CFU/g)

The results show that the water content of the *C. mangga* extract (3.71%) met the criteria of The National of Drug and Food Control. Analysis of yeast and total plate count also needs to be done in order to ensure that the extract had not contaminated by yeast and pathogenic microbes. The yeast and total plate count of the *C. mangga* extract were 0.745×10^{-4} (CFU/g) and 0.595×10^{-4} (CFU/g), respectively. According to traditional medicine quality requirements of The National of Drug and Food Control, the total plate count must not exceed 10^6 CFU/g and the yeast mold count must be below 10^4 CFU/g (15). The determination of total ash content was performed to measure the non-organic ash content after the graying process. The extract was heated until the organic compound and its derivatives were destructed and evaporated until only the inorganic composition remains. The standardization of *C. mangga* extract also includes the determination of the maximum limit of heavy metals allowed in the extract. The heavy metals like Pb and Cd were not found in *C. mangga* extract. In addition, the extract has also met the requirement of Pharmacopoeia Herbal (23).

Cell viability assay

MTT test was carried out to determine the viability of RAW 264.7 cells after exposure to plant extracts. The cells were viable ($\geq 90\%$) at concentrations ranging from 12.5 to 50 $\mu\text{g/mL}$. The results indicated that the extracts were nontoxic at the concentration tested. The viable cells were demonstrated by formazan formation which has a purple color (24).

Cytokines gene expression

The investigation on IL-6, IL-1 β , and TNF- α gene expression revealed that the extract reduced the IL-1 β , IL-6, and TNF- α expression on RAW 264.7 macrophages as compared to LPS-induced cells (Fig. 1).

DTH Response

Figure 2 shows that the footpad thickness after treatment with different doses of *C. mangga* was higher than those of negative control on normal and cyclophosphamide-induced groups ($P < 0.05$), signifying that they were increasing the DTH response and thus stimulating cell-mediated immunity.

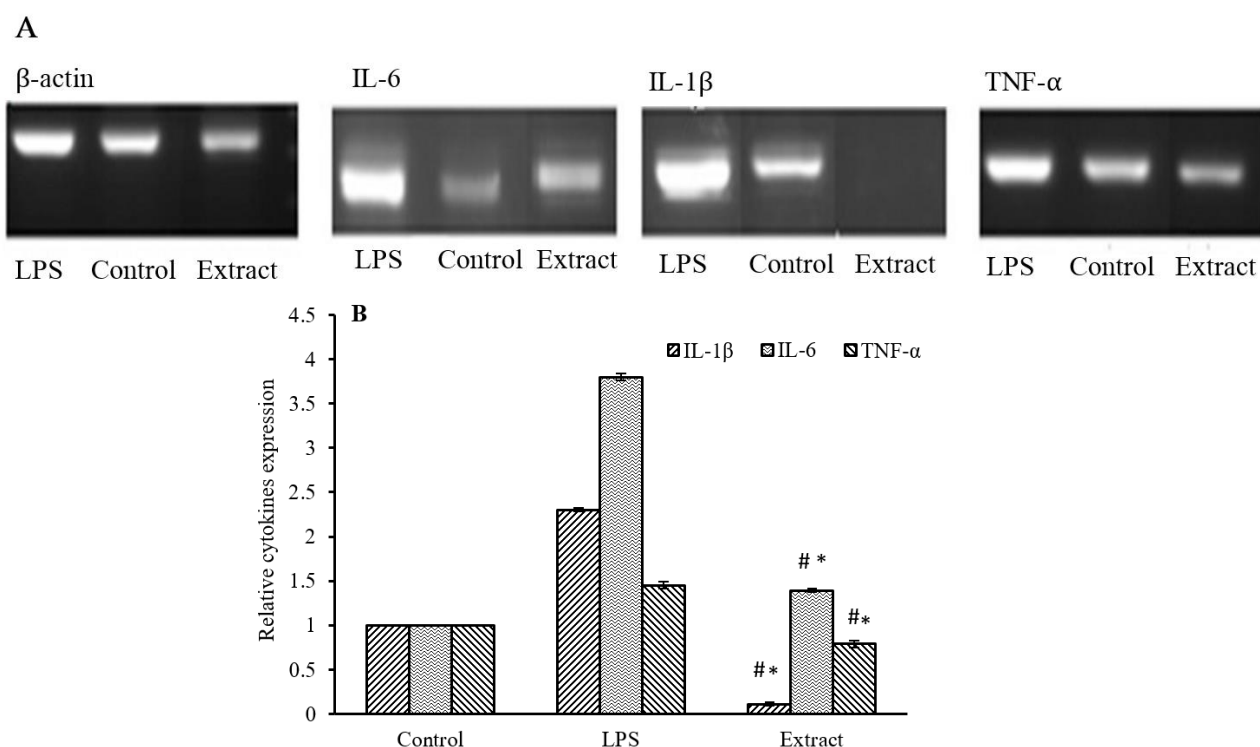


Fig. 1. (A) The effect of *Curcuma mangga* extract on the cytokines expression of genes in RAW 264.7 cells which were induced by LPS 1 $\mu\text{g/mL}$ for 6 h. (B) Densitometric analysis for cytokines expression, data present mean \pm SD, n = 3. * $P < 0.05$ Indicates significant differences compared to LPS-induced cells and # $P < 0.05$ vs the control group. IL, Interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide.

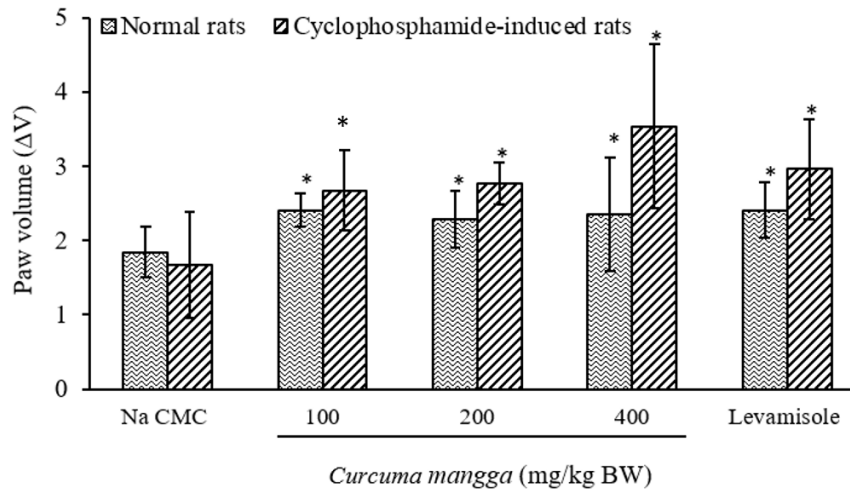


Fig. 2. The effect of ethanol extract of *Curcuma mangga* on delayed-type hypersensitivity response of normal and cyclophosphamide induced rats. Data represent mean \pm SD, n = 5. * $P < 0.05$ Indicates significant differences compared to the corresponding negative control group (Na CMC 0.5%). Levamisole was used as a positive control at 25 mg/kg BW. Na CMC, Sodium carboxymethyl cellulose.

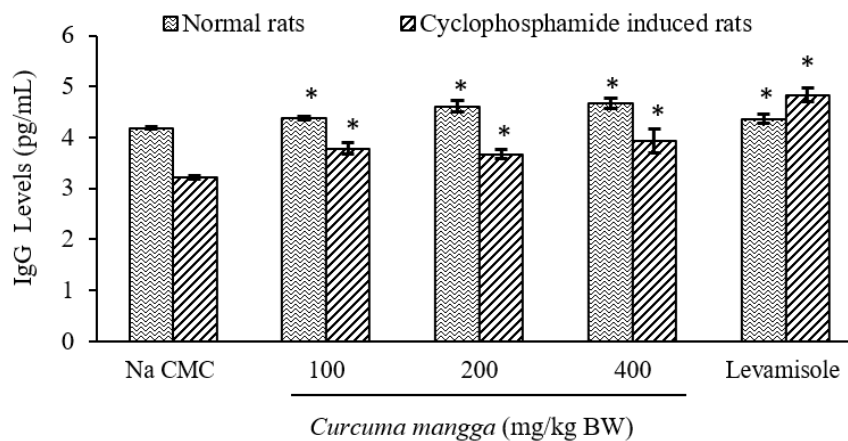


Fig. 3. The effect of *Curcuma mangga* extract on IgG production from normal and cyclophosphamide-induced rats. Data are presented as mean \pm SD, n = 5. * $P < 0.05$ Indicates significant differences compared to the corresponding negative control group (Na CMC 0.5%). Levamisole was used as a positive control at 25 mg/kg BW. Na CMC, Sodium carboxymethyl cellulose; IgG, immunoglobulin.

Immunomodulatory assay on antibody and cytokine production

Figure 3 shows that the ethanol extract of *C. mangga* increased the production of IgG in rats after infected by *S. typhimurium* which was comparable with those of levamisole (IgG levels of 4.66. and 4.36 pg/mL for *C. mangga* at 400 mg/kg BW and levamisole, respectively). The data was supported by the enhancement of IgG production from immunosuppressed rats. In contrast, the extract

revealed inhibition on IL-4 production as shown in Fig. 4.

C. mangga inhibited the production of IL-4 in a dose-dependent manner. Of all the samples, the ethanol extract of *C. mangga* at a dose of 400 mg/kg BW demonstrated the strongest inhibition with IL-4 level of 49.35 pg/mL. Cyclophosphamide suppressed the IL-4 production, furthermore the extract reduced the IL-4 levels after treatment for 14 days.

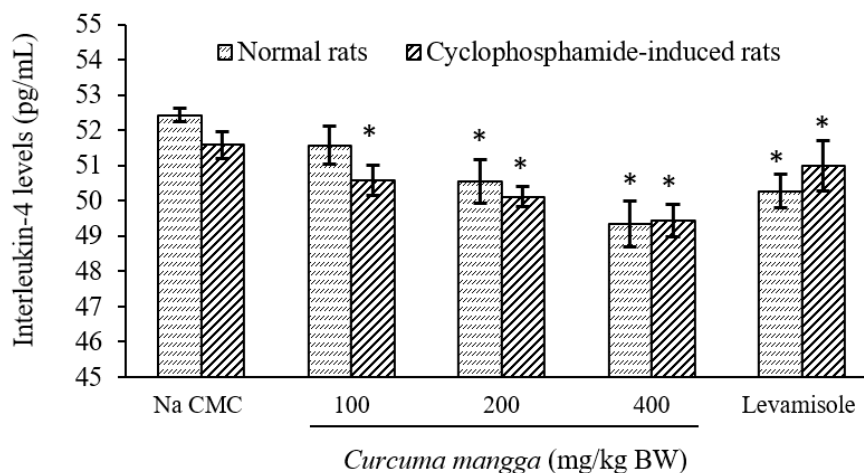


Fig. 4. The effect of *Curcuma mangga* extract on interleukin-4 production from normal and cyclophosphamide-induced rats. Data are presented as mean \pm SD, n = 5. * $P < 0.05$ Indicates significant differences compared to the corresponding negative control group (Na CMC 0.5%). Levamisole was used as a positive control at 25 mg/kg BW. Na CMC, Sodium carboxymethyl cellulose.

DISCUSSION

The ethanol extract of *C. mangga* met the requirement of the National of Drug and Food Control, Indonesia. The immunomodulatory activities of ethanol extract of standardized extract of *C. mangga* rhizomes were evaluated *in vitro* using RAW 264.7 macrophages, meanwhile the *in vivo* study was performed using *S. typhi* infected Wistar rats. The extract inhibited the gene expression of IL-1 β , IL-6, as well as TNF- α as compared to LPS-induced cells. Cytokines such as TNF- α , IL-6, and IL-1 β play an important role in regulating acute-phase inflammatory response (25). These cytokines have a synergistic relationship, in which TNF- α acted in synergy with IL-1 β to increase IL-6 secretion (26). The modulation of these cytokines by *C. mangga* extract may prevent inflammatory reactions. The results were in agreement with the previous study which reported the ability of steroids to inhibit the gene expression of TNF- α , IL-6, IL-1 β , cyclooxygenase 2, and inducible nitric oxide synthases (27,28). A previous study reported that the extract of *C. mangga* contains terpenoids, and steroids, as well as other compounds that might have anti-inflammatory effects such as flavonoids, saponins, and glycosides (11). This report supports the inhibition of IL-1 β , IL-6, and TNF- α gene expressions by *C. mangga* extract. Therefore, it was assumed that inhibition cytokines gene

expression in RAW 264.7 macrophage cells by *C. mangga* extract decreases the production of cytokines in the human body. This cytokine inhibition also promotes anti-inflammatory effects or shorten the inflammation phase in wound healing. The results were in accordance with a previous study which reported the anti-inflammatory activities of compounds (demethoxycurcumin, 15,16 bisnorlabda-8(17), 11-dien-13-one, (E)-15,15-diethoxyabda-8(17),12-dien-16-al, and bisdemethoxycurcumin) from *C. mangga* on nitric oxide and prostaglandin E2 release using RAW 264.7 cells (15).

The *in vivo* studies on immune response were conducted to investigate the immunomodulatory effects of *C. mangga* extract on DTH response, cytokines, and antibody production. Wistar rats were used and divided into two groups, including normal rats and cyclophosphamide-induced rats. Cyclophosphamide was used to suppress the immune responses of test animals in order to evaluate the ability of *C. mangga* extract to enhance the host defense in immune-suppressed conditions. Cyclophosphamide is widely used as an anticancer agent and also used to treat autoimmune diseases. This drug can suppress the immune response by acting on both cyclic and intermitotic cells, thus causes depletion of immune cells (29). In this study, cyclophosphamide suppressed the immune responses, especially in IgG production. All

animals in this study were injected with *S. typhimurium*, a Gram-negative bacterium that can cause typhoid fever in humans (30). The ethanol extract of *C. mangga* was able to enhance the paw volume of rats after infected with *S. typhimurium*. The enhanced paw edema indicates the elevated-DTH response of rat immune cells. The extract was also able to enhance the DTH response of immune-suppressed rats, indicating the immunostimulatory activity of *C. mangga* extract. The result was in accordance with the previous study which reported the immunostimulatory activity of *C. mangga* extract on phagocytosis response (11). In contrast, the *C. mangga* extract reduced the gene expression of inflammatory cytokines and decreased IL-4 production. A previous study has reported the role of IL-4 as a regulator of Th1 responses and may suppress DTH response (31). The results indicate that the inhibitory effect on IL-4 production may contribute to the enhancement of DTH response after treatment with *C. mangga* extract. Furthermore, the investigation on antibody production demonstrated that *C. mangga* increased IgG production from normal and immune-suppressed rats. Of all the samples, *C. mangga* extract at 400 mg/kg BW displayed the highest stimulation on IgG production. However, the extract reduced IL-4 production in normal and cyclophosphamide-induced groups. The result was in agreement with cytokines gene expression evaluation. Levamisole, the positive control of this study was also found to downregulate IL-4 messenger RNA (32). IL-4 is a cytokine that participates in B cell and T cell differentiation (33). It plays a major role in inducing the production of IgG1 and IgE isotypes (34). In this study, the extract increased IgG production and decreased IL-4 production indicating, that the extract stimulated the IgG production without the aid of IL-4, and another pathway may be contributed. It is known that antibodies can be produced from B cells through two different pathways of T-dependent and T-independent. Antibody production by T-dependent response requires stimulation from CD4⁺ T cells by releasing IL-4 while T-independent antigens stimulate Ig production by B cells without T

cell involvement. T independent responses are divided into type I and type II. T-independent type II immune response produces memory B cells whose secondary activation is regulated by antigen-specific IgG antibodies. Thus, the stimulation of IgG production by the extract might be due to activation of T-independent immune response (35).

CONCLUSION

The standardized extract of *C. mangga* was able to regulate the immune response, especially on the cytokines gene expression, DTH response, antibody, and cytokine production. The extract inhibited IL-6, IL-1 β , as well as TNF- α gene expression. Further investigation by *in vivo* study demonstrated that *C. mangga* extract enhanced DTH response and IgG production. The result was supported by the elevation of DTH response and IgG production from cyclophosphamide-induced immune-suppressed rats. Evaluation of IL-4 production from normal and cyclophosphamide-induced immune-suppressed rats revealed that the extract decreased IL-4 production. The results indicated that ethanol extract of *C. mangga* rhizomes has the potential to be developed into a standardized immunomodulation product. However, further studies are required to elucidate its mechanism in different lineages of the immune response.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

Yuandani designed and supervised the study, drafted the manuscript, and gave the final approval of the manuscript to be submitted for publication. S.E. Nugraha and D. Satria conducted the experiments and analyzed the data. L. Laila analyzed the data and revised the manuscript.

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