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Short Communication: Immunostimulatory effect of *tempoyak* (fermented durian) on inducing cytokine production (IL-6 and TNF-α) by RAW 264.7 cells

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Tel.: +62-21-29567888 ♥email: s.susanto@i3l.ac.id
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Abstract. *Susanto S, Sumarpo A, Parikesit AA, Putra ABNN, Ishida E, Tabuchi K, Sugahara T. 2018. Short Communication: Immunostimulatory effect of* tempoyak *(fermented durian) on inducing cytokine production (IL-6 and TNF-α) by RAW 264.7 cells. Biodiversitas 19: 318-322.* Indonesia is known to be home to various fermented foods with many-reported usage as potential sources of probiotics. *Tempoyak* (fermented durian) is among one of the Indonesian fermented foods that are rarely studied for its bioactivities. This study was conducted to evaluate the potential bioactivities of *tempoyak*, particularly the immunostimulatory aspects. Water extract of *tempoyak* was prepared by suspending the freeze-dried *tempoyak* sample in distilled water. Immunostimulatory activity of *tempoyak* water extract was evaluated using mouse macrophage cell line RAW 264.7. ELISA was used to screen cytokine productions (IL-6 and TNF-α) by RAW 264.7 cells following treatment with *tempoyak* water extract. In addition, real-time RT-PCR was also used to determine IL-6 and TNF-α mRNA expression. We showed that water extract of *tempoyak* exerts immunostimulatory effects towards RAW 264.7 cells. This was observed from the increased production of IL-6 and TNF-α in a dose-dependent manner. This was also supported by increased IL-6 and TNF-α mRNA expression. Our finding suggests that *tempoyak* has immunostimulatory effects towards murine macrophage cell line RAW 264.7. However, further studies are needed to identify the specific compounds responsible for inducing immunostimulatory effects.

Keywords: Cytokine production, IL-6, immunostimulatory, *tempoyak,* TNF-α

INTRODUCTION

Indonesia is known for various fermented foods with, most of them have been reported as potential sources of probiotics. It is suggested that the probiotics activity of fermented food is due to the presence of lactic acid bacteria (LAB) in the fermented food. LAB is long believed to possess several potential nutritional or health benefits, including improved nutritional value of food, control of intestinal infections, improved digestion of lactose, and control of serum cholesterol levels (Gilliland 2006). Moreover, fermented food is also known to promote immune responses that protect human body from invading pathogens (Parvez et al. 2006). The following health benefits may result from either probiotic effect of the food, interaction between the ingested microbes with the host, or biogenic effect of the food, which is a result of the interaction with the ingestion of microbial metabolites produced during the fermentation process (Stanton et al. 2005).

Some of Indonesian fermented foods include *oncom* (fermented soybean), *tape* (fermented cassava), *brem* (fermented glutinous rice), and other various kinds of fermented foods. *Tempoyak* (fermented durian) is among one of Indonesian fermented food that is rarely investigated. The fermentation process of *tempoyak* itself does not involve direct addition of LABs; however it relies on natural lactic acid fermentation, by salt addition into the durian pulp, which might inhibit the growth of pathogenic microorganisms, and promotes the growth of LABs (Leisner et al. 2001; Neti et al. 2011). This particular fermented food is known to contain a number of LABs, which falls under the species of *Lactobacillus, Enterococcus, Weisella,* and *Pediococcus* (Nuraida 2015). *Lactobacillus plantarum* is among one of the predominant LAB in *tempoyak* and reported to exhibit health beneficial properties when ingested in an appropriate amount (Leisner et al. 2001; Neti et al. 2011). This could be due to the production of metabolites compound by the LABs during the fermentation process. However, the health benefits of these metabolites produced during the fermentation process are still elusive.

Macrophages are classified as phagocytic cells and are considered as one of the most important cells in the innate immune system (Ishida et al. 2017). In the presence of foreign microbial pathogens, macrophages are activated, and this induces their migration to the site of infection. Pattern-recognition receptors (PRRs) are present in the

surface of macrophages, and these receptors mediate the recognition of microbial pathogens present at the site of infection. The receptors are able to recognize specific molecular structure present on the surface of pathogens identified as pathogen-associated molecular patterns (PAMPs). In addition, macrophages can also recognize damage-associated molecular patterns (DAMPs) that are often present in dead cells (Takeuchi and Akira 2010). The activation of macrophage via recognition of either PAMPs or DAMPs mediated by PRRs, consequently leads to the activation of several immune-response related downstream signaling cascades in macrophages (Heinrich et al. 2003). This event leads to the secretion of pro-inflammatory cytokines by macrophages, which include interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). The secretion of these cytokines mediates the initiation of various immune responses that help to protect human body from deleterious effects exerted by invading pathogens. In this study, the immunostimulatory effects of *tempoyak* are evaluated on innate immune system by using mouse macrophage-like cell line RAW264.7 cells.

MATERIALS AND METHODS

Procedures

Cells and cell culture

RAW 264.7 cells, a mouse macrophage-like cell line, were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). RAW 264.7 cells were maintained in DMEM supplemented with 100 U mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin, and 10% FBS at 37ºC in a humidified environment containing 5% CO_2. Phosphate buffer saline (PBS) containing 0.25% trypsin and 0.02% ethylenediamine-N,N,N',N'-tetraacetic acid (Dojindo Laboratories, Kumamoto, Japan) was used to detach RAW 264.7 cells.

Sample preparation

Tempoyak samples were purchased from two different suppliers, one was purchased from a traditional market located in Palembang, Indonesia, and the other one was purchased from an online distributor based in Palembang, Indonesia. The *tempoyak* purchased from traditional market was abbreviated as fermented durian traditional (FDT), while the other *tempoyak* purchased from online distributor was abbreviated as fermented durian online (FDO). Both *tempoyak* samples were freeze-dried for 24 h and afterward grounded into powder form. The powdered *tempoyak* samples were then suspended in deionized distilled water (DDW) at 0.1 g mL^{-1} and stirred at 12ºC for 24 h. The liquefied samples were centrifuged two times at 12,000 rpm, 4ºC for 20 min and 70,000 rpm, 4ºC for 20 min respectively. After centrifugation, the supernatant was collected and dialyzed using a 500 Da dialysis membrane (Wako Pure Chemical Industries) against DDW overnight at 4ºC. The dialyzed supernatant was then filtered through 0.22 μm filter and *tempoyak* water extract was obtained. *Tempoyak* water extract was then stored at-20ºC until subsequent use.

Cell viability assay

RAW 264.7 cells suspended in 10% FBS-DMEM medium was seeded into a 96-well culture plate (Corning) at 3.0 x 10^5 cells mL^{-1} and cultured at 37ºC for 16 h under humidified 5% CO_2. The medium was then aspirated and the cells were washed with PBS. After washing step, the cells were then treated with 200 μL of 10% FBS-DMEM medium containing various concentrations of *tempoyak* water extract or deionized distilled water (DDW) as control group and incubated at 37ºC for 6 h. After 6 h of incubation, the culture media was aspirated, and cell viability was measured using WST-8 solution (Nacalai Tesque) according to the manufacturer's instructions.

Cytokine production assay

RAW 264.7 cells suspended in 10% FBS-DMEM medium was seeded into a 96-well culture plate (Corning) at 3.0 x 10^5 cells mL^{-1} and cultured at 37ºC for 16 h under humidified 5% CO_2. The medium was then aspirated, and the cells were washed with PBS. After washing, the cells were then treated with 200 μL of 10% FBS-DMEM medium containing various concentrations of *tempoyak* water extract or DDW as control group and incubated at 37ºC for 6 h. After incubation, the culture media was collected, and IL-6 and TNF-α concentrations in the culture media were measured by ELISA using Mouse IL-6 ELISA MAX standard kit (BioLegend) and TNF-α Mouse Uncoated ELISA Kit (Invitrogen), respectively according to the manufacturer's instructions.

Real-time RT-PCR

RAW 264.7 cells suspended in 10% FBS-DMEM were seeded into a 35 mm culture dish (Falcon) and cultured for 16 h at 37ºC under humidified 5% CO_2. The medium was then aspirated, and the cells were washed with PBS. The cells were then treated with 2 mL of 10% FBS-DMEM medium containing *tempoyak* water extract at the final concentrations of 189 and 105 μg/mL or DDW as control group and incubated at 37ºC for 3 h. After incubation for 3 h, total RNA was isolated from the cells using Sepasol-RNA I Super G (Nacalai Tesque) according to the manufacturer's instructions. The isolated total RNA was used as template for cDNA synthesis using M-MLV reverse transcriptase (Promega) and an oligo-$(dT)_{20}$ primer (Toyobo). Afterwards, the synthesised cDNA was subjected to real-time RT PCR, performed using StepOnePlus Real-Time PCR System (Applied Biosystems). A 20 μL of real-time RT PCR mixture was prepared composing of 10 μL of Thunderbird SYBR qPCR Mix (Toyobo), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 6 μL of ultrapure water, and 2 μL of cDNA sample (10 ng mL^{-1}). β-actin was used as the endogenous control. The thermal cycling conditions were as follows: 20 s at 95ºC, 40 cycles of 3 s at 95ºC and 30 s at 60ºC. The primers for each specific genes are shown in Table 1.

Table 1. Sequences of primers for real-time RT-PCR

Primer	Sequences (5'-3')
Mouse β-actin	CATCCGTAAAGACCTCTATGCCAAC (sense)
	ATGGAGCCACCGATCCACA (antisense)
Mouse TNF-α	CTACTCCCAGGTTCTCTTCAA (sense)
	GCAGAGAGGAGGTTGACTTTC (antisense)
Mouse IL-6	AAGCCAGAGTCCTTCAGAGAGAT (sense)
	TTGGATGGTCTTGGTCCTTAGC (antisense)

Statistical analysis

Each result is expressed as the mean the mean ± standard deviation (SD). Student t-test was used to assess the statistical significance of the difference. Each value of * $p < 0.05$ and ** $p < 0.01$ is considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of *tempoyak* water extract on RAW 264.7 cells viability

Cell viability assay was carried out to determine cytotoxicity of *tempoyak* water extract towards RAW 264.7 cells. RAW 264.7 cells were treated with culture medium containing *tempoyak* water extract at various concentrations. After 6 h of incubation, the culture media was aspirated, and cell viability was measured using WST-8 solution. As shown in Figure 1, cell viability assays show that *tempoyak* water extracts do not induce cytotoxicity towards RAW 264.7 cells even at the maximum concentration.

Effect of *tempoyak* water extract on IL-6 and TNF-α production by RAW 264.7 cells

The effect of *tempoyak* water extract on cytokine production by RAW 264.7 cells was examined. RAW 264.7 cells were treated with culture medium containing *tempoyak* water extract at various concentrations. The amount of IL-6 and TNF-α concentration was measured after 6 h of incubation by ELISA. Result from ELISA experiment shows that the production of IL-6 and TNF-α concentration in RAW 264.7 cells treated with *tempoyak* water extract increases in a concentration-dependent manner (Figure 2). RAW 264.7 cells were treated with 100 μg mL^{-1} of FDO water extract, the production of IL-6 and TNF-α was increased by 33.9-fold and 80.9-fold, respectively when compared to control. On the other hand, RAW 264.7 cells treated with 100 μg mL^{-1} of FDT water extract, the production of IL-6 and TNF-α was increased by 16.5-fold and 50.7-fold, respectively when compared to control; but slightly lower when compared with FDO treated group. Amount of TNF-α production in both FDO and FDT water extract treated groups were significantly upregulated compared to IL-6 production, suggesting both samples were more potent in stimulating TNF-α expression. Cytokine production was not observed in RAW 264.7 cells treated with fresh durian water extract.

Effect of *tempoyak* water extract on gene expression levels in RAW 264.7 cells

As previously discussed, *tempoyak* water extract induced IL-6 and TNF-α expression in RAW 264.7 cells. To investigate, the effect of *tempoyak* water extract on mRNA expression of IL-6 and TNF-α was examined. *Tempoyak* water extracts, FDO and FDT, were added to cell culture medium at 100 μg mL^{-1}, and mRNA expression levels of IL-6 and TNF-α were determined by real-time RT-PCR. Both *tempoyak* water extract, FDO, and FDT significantly increase mRNA expression levels of IL-6 and TNF-α in RAW 264.7 cells. The mRNA expression levels of IL-6 and TNF-α in RAW 264.7 cells treated with FDO were significantly increased by 61.9-fold and 3.7-fold, respectively, compared to control. Furthermore, mRNA expression levels of IL-6 and TNF-α in RAW 264.7 cells with FDT was increased by 22.9-fold and 2.7-fold, respectively, compared to control. Taken together, our results suggest that *tempoyak* water extract induces mRNA expression level of IL-6 and TNF-α in RAW 264.7 cells by upregulating cytokine gene expression.

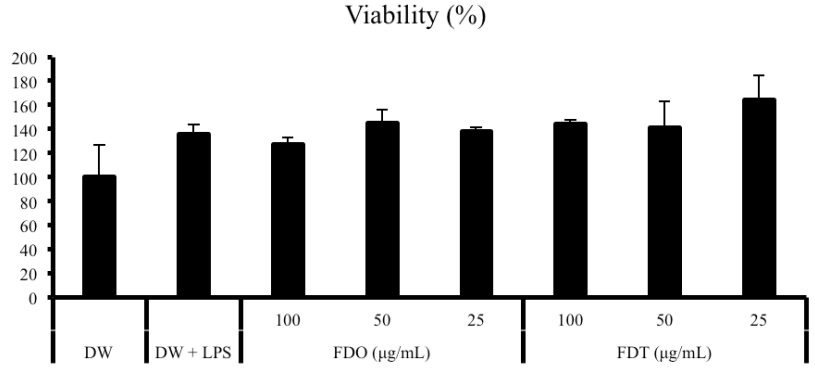

Figure 1. Effect of *tempoyak* water extract on RAW 264.7 cells viability. RAW 264.7 cells were treated with culture medium containing *tempoyak* water extract at various concentrations. After 6 h of incubation, the culture media was aspirated and cell viability was measured using WST-8 solution. Negative control cells were cultured in 10% FBS-DMEM medium containing DDW, while positive control cells were treated in 10% FBS-DMEM medium containing lipopolysaccharide (LPS). The data represent the mean ± SD of triplicate experiments.

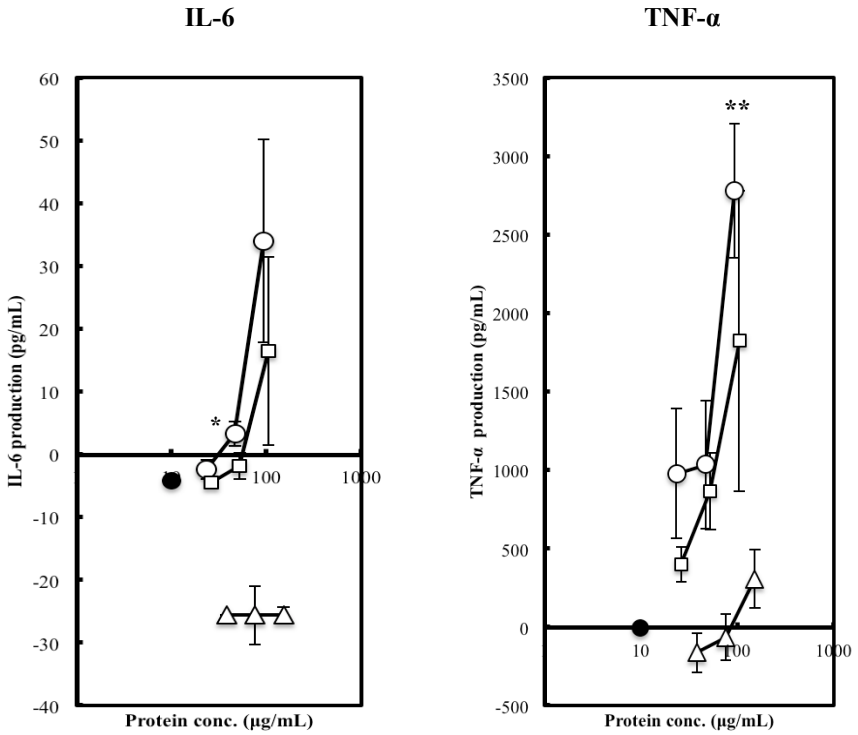

Figure 2. Effect of *tempoyak* water extract on cytokine production in RAW 264.7 cells. RAW 264.7 cells were cultured in 10% FBS-DMEM medium containing varying concentration of *tempoyak* water extract (FDO and FDT). Control cells were cultured in 10% FBS-DMEM medium containing DDW. The cell was incubated for 6 h at 37°C. ELISA was used to determine the cytokine concentration in culture media. The data represent the mean±SD of triplicate experiments. Close circle: control; open circle: FDO; open square: FDT; open triangle: fresh durian. Statistical analysis was done using student t-test $^{*}p < 0.05$ and $^{**}p < 0.01$.

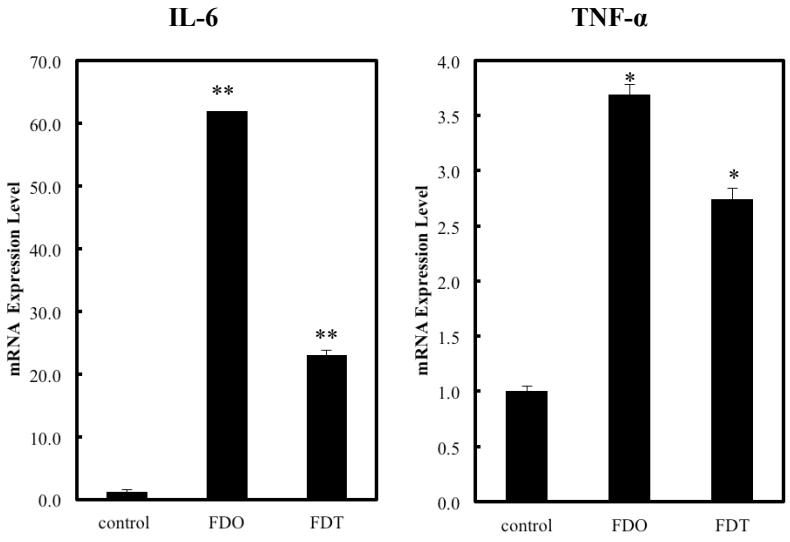

Figure 3. Effect of *tempoyak* water extract on gene expression levels in RAW 264.7 cells. RAW 264.7 cells were cultured in 10% FBS-DMEM medium containing FDO and FDT water extract with concentration 100 µg mL^{-1}. Control cells were cultured in 10% FBS-DMEM medium containing DDW. Cells were incubated for 3 h at 37°C. Total RNA was isolated from the cell, and real-time RT-PCR was carried out to determine gene expression levels. The data represent the mean±SD of duplicate experiments. Statistical analysis was done using student t-test $^{*}p < 0.05$ and $^{**}p < 0.01$

Discussion

The immunostimulatory properties of fermented foods have long been reported. Various studies involving various kinds of fermented foods have been conducted in order to provide scientific evidence on the immunostimulatory effects of fermented foods. It is speculated that there were two possible factors that underlie the immunostimulatory effects of fermented foods (Perdigón et al. 2002; Granier et al. 2013). Immunostimulatory effects exerted by fermented foods might be due to the presence of fermenting bacteria contained in the food, presumably LABs, or the effect could also be a result of metabolite product generated during fermentation process (Perdigón et al. 2002; Granier et al. 2013).

Generation of pro-inflammatory cytokines such as IL-6 and TNF-α marks the activation of macrophage, which is an essential response of the immune system to invading pathogens (Wijanarti et al. 2015). Secretion of TNF-α by macrophages induces the transcription of genes that are responsible for encoding other inflammatory molecules (Parameswaran et al. 2010). These molecules are considered to contribute in the priming process of macrophages that generates enhanced responsiveness towards pathogens invasion (Parameswaran et al. 2010). In addition to its role in enhancing macrophages response, TNF-α also plays a role in the mediating the recruitment of other immune cells such as lymphocytes which enhances immune response (Wijanarti et al. 2015). Similar to TNF-α, IL-6 also modulates the responsiveness of the immune cell towards pathogen invasion, particularly by mediating macrophages polarization (Fernando et al. 2014). Macrophages are known as innate immune cells that possess two distinct phenotypes which are a pro-inflammatory subset that is associated with and an anti-inflammatory subset (Mosser and Edwards 2008). IL-6 is known to enhance the polarization of macrophage towards the anti-inflammatory subset, thereby promoting anti-inflammatory benefits of macrophage (Fernando et al. 2014). In addition to mediating macrophage polarization, IL-6 also plays a role in mediating B cell differentiation into plasma cells (Wijanarti et al. 2015).

In the following *in vitro* study described here, the immunostimulatory effects of *tempoyak* were investigated by observing the immunostimulatory effects that were exerted towards mouse macrophage cell line RAW 264.7. The results of the study demonstrated the ability of *tempoyak* to induce IL-6 and TNF-α production by RAW 264.7 cells in a concentration-dependent manner relative to control group. Interestingly, the immunostimulatory properties possess by *tempoyak* water extract largely originates from the fermentation process; cytokine production was hardly observed in RAW 264.7 cells treated with fresh durian. Therefore, this finding suggests that the fermentation process of *tempoyak* might generate bioactive compounds that are responsible for the immunostimulatory effects. Therefore, further investigation is necessary to determine its identity. The following result suggests that *tempoyak* possess potential immunostimulatory properties and therefore could be utilized as a potential candidate for processed food.

Taken together, *tempoyak* water extract is shown to have potential immunostimulatory properties. This was shown through the ability of *tempoyak* water extract to induce cytokine production in RAW 264.7 cells. Moreover, the immunostimulatory properties possessed by *tempoyak* are largely due to the fermentation process. There is a possibility that during fermentation process, the bacteria produce specific metabolites that could potentially be responsible for the immunostimulatory properties found in *tempoyak*. Further investigation is necessary to investigate the bioactive compounds capable of inducing immunostimulatory effect in *tempoyak*.

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