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The Effect of Fermented Soybean Extract Supplementation on Pro Inflammatory Cytokines among *Rattus Norvegicus* Infected by *Mycobacterium Tuberculosis*

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ABSTRACT

Objective of the study was to investigate the effectiveness of extract tempeh supplement on proinflammatory cytokines profile in Wistar rats infected by the endotracheal route with *Mycobacterium tuberculosis*. The study design was a randomized, post-test only, control group. The ethanolic extract tempeh supplement at concentrations of 200, 400, and 800 mg/kg body weight were orally administered daily for 14 consecutive days to three experimental groups. The control group received carboxymethyl cellulose sodium. Another group was to be sacrificed to confirm the development of active pulmonary Tuberculosis (TB) infection using histopathological evaluation. Serum concentrations of tumor necrosis factor-alpha (TNF- α), interferon gamma (INF- γ), and interleukin (IL)-2, IL-6, IL-10, and IL-12 were measured by enzyme-linked immunosorbent assay (ELISA) method. There were no significant differences in the levels of TNF- α , IL-2, IL-6, IL-10, and IL-12 at all concentrations compared to control group. At the concentration of 800 mg/kg body weight supplementation, the level of INF- γ was significantly lower than control ($p = 0.0047$). In the animal model of tuberculosis, the study showed that supplementation with different concentrations of ethanolic tempeh extract had no significant impact on proinflammatory cytokines.

Keywords: *Mycobacterium tuberculosis*, proinflammatory cytokines, tempeh, tuberculosis

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1. Introduction

Tuberculosis (TB) is a contagious disease that often affects the lungs (pulmonary TB), though it can also attack other organs (extrapulmonary TB). It is caused by an airborne pathogen, acid-fast, rod-shaped, and aerobic bacteria, called *M. tuberculosis*. The disease spreads from an infected person to a healthy one through the aerosol transmission of droplets containing the bacteria, for example, by coughing, sneezing, shouting, or singing both within the household and outside (1-3). According to the World Health Organization, TB is one of the top 10 causes of death and the leading cause of a single infectious agent globally. With an estimated 1.3 million deaths in 2017 among HIV-negative people and an additional 300.000 deaths from TB among HIV-positive people, millions of people continue to fall sick with TB each year. Globally, the best estimate is that 10.0 million people developed TB disease in 2017 (4).

In individuals with an adequate immune system, the subsequent defense mechanism is to form granulomas around the *M. tuberculosis* (5). These nodular-type lesions are formed by macrophages and activated T lymphocytes to create conditions that prevent further mycobacteria growth (6). The lesions have a caseous necrotic environment with a lack of resources for their multiplication (7). Later on, it can be either latent tuberculosis or active progression, known as primary progressive tuberculosis (8,9).

Studies indicate a significant correlation in the increase in Interleukin-6 (IL-6) against the acute phase response in TB patients compared to controls, especially those who experienced thrombocytosis. Increased IL-6 levels are also associated with increased degrees of phlegm removal, lesions on chest X-ray, and the severity of symptoms suffered in active pulmonary TB (10). *M. tuberculosis* induces secretion of TNF- α by macrophages, dendritic cells, and T cells (11,12). One of the cytokines produced by T helper cells (Th1) is gamma interferon (IFN- γ), which plays an important role in eliminating the bacteria *M. tuberculosis*. Likewise, with IL-10, various studies have identified that IL-10 correlates with susceptibility to tuberculosis, both in humans and in experimental animals (rats).

Soy isoflavones are a source of phytoestrogens in the diet of people. Soy isoflavones have been found to downregulate proinflammatory cytokines and exert anti-inflammatory activity due to their Peroxi-

some Proliferator-Activated Receptor alpha/gamma (PPAR α/γ) agonists properties (13). In vitro studies using various types of cell lines indicate that isoflavones can provide a downregulation effect of pro-inflammatory cytokines IL-6 and TNF- α (14,15). Soybeans consumed directly as food by postmenopausal women with metabolic syndrome can reduce markers of inflammatory nitric oxide (NO), serum E selectin, IL-18, and C-Reactive Protein (CRP), even better than soy extract powder. A study involving 1,005 participants showed that consumption of foods made from soy reduced levels of IL-6, TNF- α , and soluble TNF receptors significantly in adult women in China (16).

Tempeh is a traditional Indonesian cuisine produced from fermented soybeans. It is frequently prepared using *Rhizopus* sp. as a starter to increase the quality of the soybeans compared to unfermented soybeans (17). Where a previous preliminary study has shown that giving *tempeh* as supplementary food for the first two months can accelerate the process of restoring nutritional status and physical strength of pulmonary TB patients (18), local soy-based food products have the potential to be a supportive nutrient to accelerate the healing process of TB patients. However, the positive effect is not directly related to the addition of calorie and protein intake. To answer this question, data is needed on the effect of extract *tempeh* provision on proinflammatory cytokine levels. As the purpose of this study was to analyze whether the administration of *tempeh* extract among male rats (*Rattus norvegicus*) infected by *M. tuberculosis* results in a positive effect on pro-inflammatory cytokine markers, it is further an attempt to provide an explanation of the mechanisms associated with the positive effects of proinflammatory cytokines from *tempeh* which can accelerate the recovery process of TB patients.

2. Material and Methods

2.1 Reagents and Chemicals

Soybeans [*Glycine max* (L.) Merr] var. *Grobogan* was obtained from a research institute for various beans and tubers in Malang, East Java, Indonesia. The *tempeh* starter containing “*Rhizopus oligosporus* and rice flour,” for the solid-state of fermentation, was purchased from the local market in Surabaya, Indonesia. The brand name of the *tempeh* starter was

Raprima™ and it was produced by Aneka Fermentasi Industri, Bandung, Indonesia. Isoflavone standards, namely genistein ($\geq 9\%$) and daidzein ($\geq 98\%$) for high-performance liquid chromatography (HPLC) ($\geq 95\%$), were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

2.2 Preparation of *Tempeh* Samples

The *tempeh* preparation was done according to the previous study by Tjandra et al. (19). Briefly, soybeans were boiled twice before fermentation. Consequently, 500 g of yellow soybeans were boiled in 100°C pre-heated tap water for 30 min. After that, the water used for boiling was discarded and the wet method was used to peel soybeans. Dehulling was done using hands to rub the hulls from cotyledons until nearly 90% of the separated skins were removed from the water. The hulls floating on the top of the water were removed during the draining of the water. Then soybean seeds were soaked overnight for 12 hours in fresh tap water with a water level 5 cm over the beans at a room temperature ranging between 27°C to 30°C. The second boiling of the soybeans was carried out in pre-heated fresh tap water at 100°C for 30 min. After water drainage, the soybeans were spread homogeneously on a flat surface with a clean fabric for cooling to room temperature. The cooling down step was 30 min before the soybean was inoculated using a starter. One gram of a commercial *tempeh* starter Raprima™, which contained *R. oligosporus*, was added and stirred gently to inoculate the soybeans uniformly. After 20 minutes of stirring, the inoculated soybeans were then transferred into sealed plastic bags that had been perforated using a toothpick. They were placed in a room at lukewarm temperature (28°C \pm 2°C) for 48 hours. After the incubation period, the fermented soybean was kept at -10°C while waiting for the next preparation.

2.3 *Tempeh* Samples Extraction

Extraction was performed based on the method of Tjandra et al. (19). *Tempeh* samples were dried at a temperature of 40 to 45°C for 24 hours. The dried *tempeh* was crushed until it became powder (60 mesh). *Tempeh* powder (0.5 g) and 70% ethanol were stirred using a vortex and then stored in a dark room for 24 hours. The filtrate was separated using centrifugation (3000 rpm), then filtered. After the first filtrate was obtained, its residue was added again with 5 mL 70%

ethanol, and then the previous process was repeated. The first and the second filtrate were mixed and then evaporated by a rotary evaporator at a temperature of 60°C. *Tempeh* extract suspension was prepared with the solution of sodium carboxymethyl cellulose (2% CMC-Na solution) prior to administration.

2.4 Measurement of Genistein and Daidzein Composition

The samples were freeze-dried at -20°C for three days and then ground into fine particles. The freeze-dried samples were ground into powder. The ground sample was stored in airtight bottles and wrapped in aluminum foil. One gram of the sample was put into a rounded bottom flask. Forty mL of 96% ethanol (Wako Chem.) and 10 mL of (2 M) hydrochloric acid (Merck) was added into the rounded bottom flask and followed by a sonication step for 20 min. The sample mixture was heated in a water bath at 100°C and refluxed for 4 hours. The mixture was cooled down and made up to 100 mL with ethanol (96%). The sample suspension was then dropped with NaOH (Wako Chem.) up to pH 4, followed by centrifugation (Universal 30RF, Tuttlingen, Germany) at 9000 g for 20 minutes and put into a vial of (HPLC). HPLC Agilent 1100 series with an autosampler and PDA (photodiode array) detector was used for this analysis. Reversed-phase column Merck LiChrospher-100 RP-18 250 \times 4 mm, 5 μ m was used. In the isocratic mobile phase, acetonitrile-acetic acid 10% was used as an eluent at the flow rate of 0.8 mL/min, 30°C. Injection volume was 5 μ L and analytes were monitored at 260 nm. All other reagents used were analytical reagent grade. The results were expressed in % Weight/Weight (w/w).

2.5 Administration of *Tempeh* Extract in TB Animal Model

Albino male Wistar rats weighing between 150–200 grams were infected by 50 μ L solution containing 108/mL of *M. tuberculosis* strain H37RV through the trachea. *M. tuberculosis* bacteria were taken from stock grown in Lowenstein-Jensen media for 2–3 weeks. The provision of *tempeh* extract in male rats (*R. norvegicus*), as many as 35 tails were infected with *M. tuberculosis* intratracheally, was divided randomly into 5 groups. Randomization was done using the www.site.randomizer.org to get random numbers. The first, second, and third groups received

treatment of ethanol extract of *tempeh* with a dose of 200 mg/kg BW, 400 mg/kg BW, and 800 mg/kg BW, while the fourth and fifth groups were negative controls (CMC-Na) and groups that were sacrificed to ensure infection. The treatment was carried out on the 30th day after the infection. The treatment of ethanol extract of *tempeh* was given for 14 days orally. During blood sample collection, rats were in terminal anesthesia. An appropriate needle was used for blood sample collection with thoracotomy. A blood sample was taken from the ventricle of the heart. The damage of rats' lung tissue was assessed based on the score of Dorman.

2.6 Cytokine Assay

Serum concentrations of IL-2, IL-6, IL-10, IL-12, TNF- α , and IFN- γ were determined in duplicates with commercially available enzyme-linked immunosorbent assay kit, Binassay Technology Laboratory). All of the serum samples were analyzed in one assay in duplicate with intra- and interassay < 10% (IL-12 sensitivity < 2 pg/mL, interassay 4.6%, intrassay 3.4%; IL-2 sensitivity < 0.1 U/mL, interassay 7.5%, intrassay 5.7%; IL-6 sensitivity < 2 pg/mL, interassay 8.0%, intrassay 6.0%; and TNF- α sensitivity < 3 pg/mL, interassay 9.9%, intrassay 5.2%).

2.7 Statistical Analysis

Results were expressed as means and standard deviations. Each sample from the soybean, *tempeh*, and ethanol extract was measured two times. The means comparison of more than two groups were analyzed by the univariate analysis of variance, followed by the LSD posthoc test. The cut-off of the statistical significance level was set at $p < 0.05$. Statistical analyses were done through the commercially available Statistical Package for Social Sciences software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

3. Result

3.1 Comparison of Genistein and Daidzein Content of Raw Soybean and *Tempeh*

The compositions of genistein and daidzein in *tempeh* ethanol extract have shown 297.34 ± 10.115 $\mu\text{g/g}$ of samples, 454.70 ± 2.89 $\mu\text{g/g}$ samples, respectively. These results were significantly lower than uncooked *tempeh* in which their genistein and daidzein levels were 325.68 ± 7.09 $\mu\text{g/g}$ samples and 536.63 ± 2.13 $\mu\text{g/g}$ samples, respectively (Table 1). While the compositions of genistein and daidzein in soybeans showed 113.33 ± 1.42 $\mu\text{g/g}$ and 291.84 ± 9.93 $\mu\text{g/g}$ samples, respectively, which were significantly lower than the content of *tempeh* extract (Figure 1) so that uncooked *tempeh* is described as having the highest content of genistein and daidzein while soybeans produce the lowest concentration.

3.2 Histopathological Assessment of Post-Infection Rats

The administration of 50 μl solution containing *M. tuberculosis* H37RV strain to *R. norvegicus* intratracheally with an incubation period of four weeks produced signs of pathological responses in the lung tissues. These signs include alveolitis, granuloma, peribronchiolitis, and perivascularitis (Table 2). While in other organs such as the liver, spleen, and kidneys, lung pathology was not found as signs of TB infection using histopathological assessment.

3.3 Cytokines Concentration of the Rats after *Tempeh* Administration

3.3.1 Interleukin (IL)-6, TNF- α , and Interferon Concentration

Serum levels for IL-6 marker at a concentration of 200 mg/kg BW (19.76 ± 2.94 ng/L) showed a tendency to be higher although it was not different from control (14.98 ± 3.88 ng/L). The increase in the con-

Table 1. The mean of genistein dan daidzein levels (Mean \pm SD).

	Genistein ($\mu\text{g/g}$ sample)	Daidzein ($\mu\text{g/g}$ sample)
Soybean	113.33 ± 1.42^a	291.84 ± 9.93^a
Uncooked tempeh	325.68 ± 7.09^c	536.63 ± 2.13^c
Tempeh extract	297.34 ± 10.115^b	454.70 ± 2.89^b

Note: Values with different superscript letters (a–c) in the same row are significantly different ($p < 0.05$)

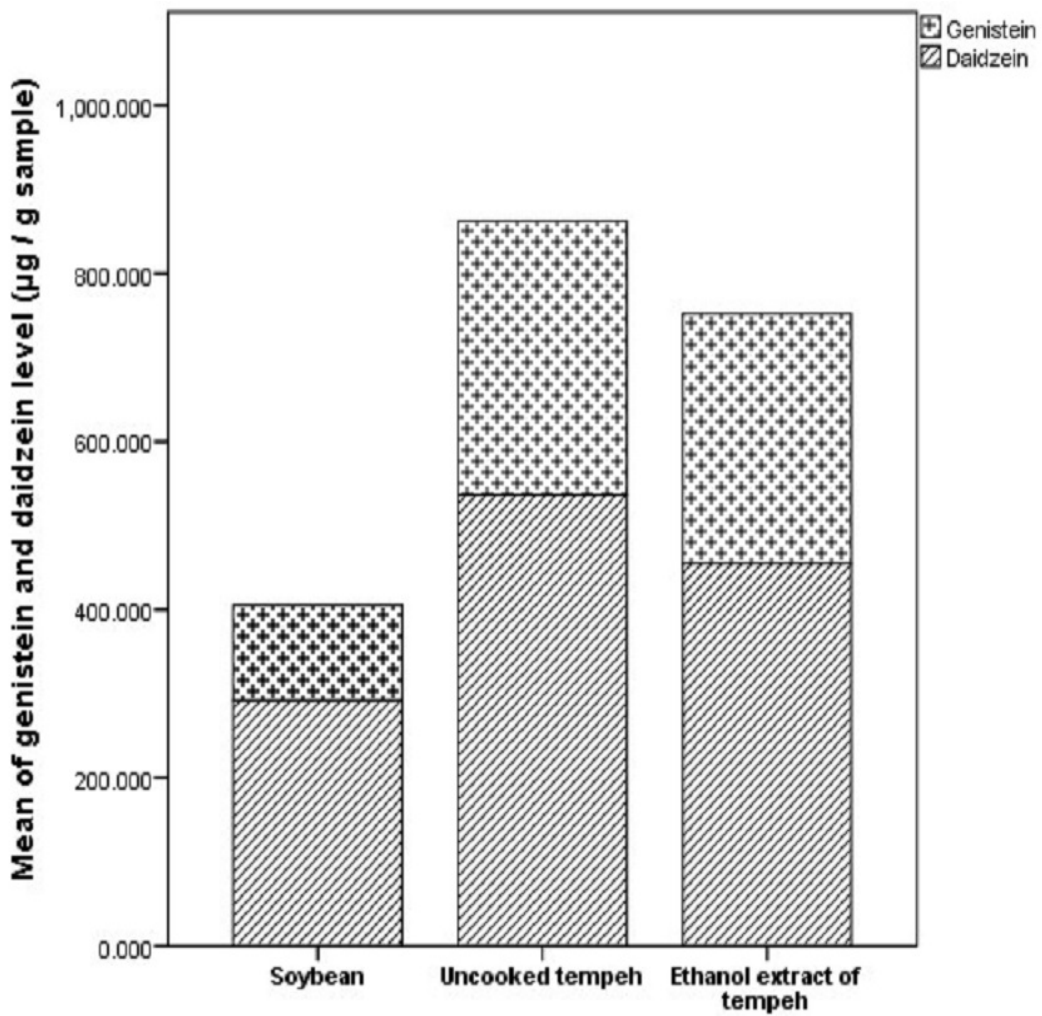


Figure 1. Evaluation of mean of isoflavones content (genistein and daidzein) in soybean, uncooked tempeh, and ethanol extract of tempeh.

Table 2. Histopathological description of *Rattus norvegicus* lung tissue infected by *M. tuberculosis*.

Rats	Alveolitis	Granuloma	Peribronchiolitis	Perivasculitis
1	3	2	3	3
2	2	1	2	2
3	4	3	4	4
4	3	2	3	3
5	2	1	2	2
6	1	1	1	1

centration of 400 mg/kg BW (20.37 ± 5.77 ng/L) was significantly higher than the control and the dose of 800 mg/kg BW (12.93 ± 4.81 ng/L) was significantly lower.

Administration of *tempeh* extract at doses of 200, 400, 800 mg/kg BW produced (4.02 ± 1.72 ng/L), (5.61 ± 1.10 ng/L), and (4.42 ± 1.26 ng/L) TNF α levels, respectively, which did not give a significant difference compared to the control (5.01 ± 0.70 ng/L). However, on IFN- γ level, administration of *tempeh* extract at a dose of 800 mg/kg BW (63.46 ± 31.53 ng/L) showed a significant difference when compared to the control (100.59 ± 17.88 ng/L), whereas doses of 200 and 400 mg/kg BW resulted in IFN γ concentrations of (98.91 ± 5.32 ng/L) and (101.24 ± 25.51 ng/L), respectively. These concentrations did not produce a significant difference compared to control (Table 3).

3.3.2 IL-2, IL-10, and IL-12 Concentration

Interleukin 2 (IL-2) serum levels after the provision of ethanolic *tempeh* extract at concentrations of 200, 400, and 800 mg/kg BW have shown (1064.34 ± 122.27 ng/L), (957.26 ± 251.07 ng/L) and (725.62 ± 330.96 ng/L), respectively. These levels did not give a significant difference compared to control (899.04

± 216.80 ng/L). However, the extract of concentration 200 mg/ kg BW showed a significant difference compared to the concentration of 800 mg/kg BW with $p = 0.015$. The administration of concentration 200, 400, and 800 mg / kg BW produced IL-10 levels in rat serum of (530.02 ± 38.89 pg/mL), (493.77 ± 100.54 pg/mL), and (412.81 ± 194.74 pg/mL), respectively. These levels were not significantly different from control (444.52 ± 87.97 pg/mL). Interleukin 12 (IL-12) levels after the administration of *tempeh* ethanol extract with concentrations of 200, 400, and 800 mg/kg BW produced the concentrations (17.37 ± 1.72 pg/mL), (16.44 ± 1.16 pg/mL), and (16.69 ± 1.85 pg/mL), respectively. These concentrations did not give a significant difference with control (17.58 ± 2.27 pg/mL) (Table 4).

4. Discussion

In this study, it has been shown that the total concentration of genistein and daidzein in *tempeh* extract was significantly lower ($p < 0.05$) than uncooked *tempeh*. While ethanol is recommended for extracting soybean antioxidants on the basis of the high total phenolic content and oxygen radical absorbance capacity results (20,21), solvents such as acetonitrile

Table 3. Histopathological description of *Rattus norvegicus* lung tissue infected by *M. tuberculosis*.

	IL-6 (ng/L) *	TNF α (ng/L) *	Interferon γ (ng/L) *
Control	14.98 ± 3.88^{ab}	5.01 ± 0.70^{ab}	100.59 ± 17.88^b
200 mg/kg BW	19.76 ± 2.94^{ac}	4.02 ± 1.72^a	98.91 ± 5.32^b
400 mg/kg BW	20.37 ± 5.77^c	5.61 ± 1.10^b	101.24 ± 25.51^b
800 mg/kg BW	12.93 ± 4.81^b	4.42 ± 1.26^{ab}	63.46 ± 31.53^a

*Mean \pm SD (n = 7); Values with different superscript letters (a–c) in the same row are significantly different ($p < 0.05$).

Table 4. Means of IL-2, IL-10, and IL-12 serum concentrations.

	IL-2 (ng/L)*	IL-10 (pg/mL)*	IL-12 (pg/mL)*
Control	899.04 ± 216.80^{ac}	444.52 ± 87.97^a	17.58 ± 2.27^a
200 mg/kg BW	1064.34 ± 122.27^c	530.02 ± 38.89^a	17.37 ± 1.72^a
400 mg/kg BW	957.26 ± 251.07^{ac}	493.77 ± 100.54^a	16.44 ± 1.16^a
800 mg/kg BW	725.62 ± 330.96^{ab}	412.81 ± 194.74^a	16.69 ± 1.85^a

*Mean \pm SD (n = 7); Values with different superscript letters (a-c) in the same row are significantly different ($p < 0.05$).

have been found to be superior to others in extracting isoflavones in soy foods (22). This shows that in the extraction procedure using 70% ethanol, soy isoflavones were not appropriately extracted and caused less efficacy of the supplement.

Most of the parameters measured have not described significantly different results among the rats. It can be due to animal model complexity. The mouse active TB model is suitable for rapid anti-TB chemical drugs evaluation, due to the homogeneous pathological change and bacteria burden. Guinea pig presents a sensitive immune response when infected, thus it is a good model for anti-TB vaccine evaluation. Monkey TB model has similar clinical signs and classic granulomas structure to that of patients, therefore it is a priority for mechanisms of disease research (23). On the other hand, the Wistar rat is a valuable model for a better understanding of host-pathogen interactions that result in the control of Mtb infection and the potential establishment of latent TB. It is also a good animal model choice for TB drug discovery due to the ease of manipulation, relatively low cost, and well-established use of rats in toxicology and pharmacokinetic analyses (24).

Among all the parameters measured, the provision of extract *tempeh* has only resulted in a decrease of interferon γ level with 800 mg/kg BW in the TB rat model. However, other recent studies have shown several beneficial effects of extract *tempeh* supplementation on diabetes in rats (25). The use of extract or isolate of soy isoflavones might give higher efficacy on inflammatory and oxidative stress response than *tempeh* extract in rats (26,27). In the animal model, soy isoflavones are able to inhibit inflammation induced by LPS (lipopolysaccharides) and decrease levels of IL-1 β , IL-6, NO, and PGE (28). In addition to animals, the administration of soy isoflavones in patients with chronic kidney disease has been found to reduce levels of CRP, IL-6, and TNF- α . A study has shown that consumption of soy isoflavone enriched bread reduced proinflammatory cytokines among men with prostate cancer (29).

In general, proinflammatory cytokine levels after the administration of extract *tempeh* have not shown significantly different results in comparison to all control groups. The results of supplementation in Wistar rats have shown irregular pattern tendency and low efficacy. Perhaps one possible explanation is that the administration of higher-level isoflavone soy food

might have a more prominent effect on proinflammatory cytokines such as IL-6 than lower isoflavone soy food (30). Since the soy isoflavones might be the key bioactive ingredients, so their concentrations need to be carefully considered. On the other hand, further data evaluation from several randomized clinical trials suggests that neither isoflavones nor soy foods affect IL-6 and TNF α (31). In another perspective, soy-based food might give a beneficial effect on inflammation markers for a particular condition such as metabolic syndrome instead of infectious disease (32). Moreover, in a complex disease such as TB, the role of these cytokines cannot be said to be either "positive" or "negative" but rather the cytokines might result in both protective and pathologic values depending upon the context.

While currently there is no reliable evidence that routine supplementation above recommended daily amounts has clinical benefits for TB, food or nutrition may improve weight gain during recovery from tuberculosis in some settings among men but there is currently a lack of evidence that they improve tuberculosis treatment outcomes. Since rats are not the normal host of Mtb proinflammatory cytokines are more important than just weight gain. Moreover, in ethanolic *tempeh* extract, the main ingredients are polyphenol compounds than macronutrients so that weight gain might be not a better option to assess.

Additional results in this study are that TB lesions were found only on the rat's lungs but not on their extrapulmonary organs. Since rats are not the natural host of Mtb, they might be more resistant than humans to Mtb infection. In a previous study, endotracheal inoculation of Mtb strain W4 with concentration ranging from 10 to 10⁴ CFU in Wistar rats could not provide evidence of infection in extrapulmonary organs such as brain, blood kidney, and liver (24). Perhaps, this was the explanation that TB histopathological changes were only found on the rat lung tissues during this study. Besides the animal's resistance, the intratracheal route of administration of H37RV Mtb could be another reason why there was no sign of TB infection on the observed extrapulmonary organs (spleen, kidney, and liver). It could be due to the following respiratory inoculation; Mtb may reside in the lungs before the dissemination of the bacteria via lymph nodes so that extrapulmonary infection could be slower than intravenous administration. Another possibility is that the systemic dissemination of Mtb to rat's internal organs could be

only in a short period due to its higher resistant nature (24). Alternatively, a Guinea pig is a suitable option for extrapulmonary TB lesion analysis purposes because of its ability to develop a blood-disseminated disease similar to that found in humans.

However, several limitations caused by the study's methodology should be noted. The use of aerosol for bacteria inoculation in the animal model might improve the study results. It is not only more precise to the natural route of the infection, but it is also the most reproducible technique for similar studies. As aforementioned, more suitable solvents for the extraction of soy isoflavone isolate application might affect the outcomes. The availability of the samples in the middle and at the end of the treatment with a longer duration might result in a better understanding.

5. Conclusions

This study showed that the oral administration of 70% ethanol extract of *tempeh* did not provide a significant decrease in serum levels of TNF α , IL-2, IL-6, IL-10, and IL-12 among male rats (*Rattus norvegicus*). The decrease in cytokine levels occurred in the administration of *tempeh* extract at a concentration of 800 mg/kg BW on the IFN γ ($p = 0.0047$). From these results, it can be concluded that the administration of *tempeh* ethanol extract shows weak efficacy against the marker of proinflammatory cytokines and tends to require large concentrations to have a beneficial effect.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Statement of Contribution of Researchers

Concept of the study-LT, KI; Data collection -LT, BS, SLU; Literature review-BS, SLU; Data analysis-BS, SLU; Writing-LT, BS, SLU; Supervision-LT, KI.

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