The effect of fermented *Glycine max* (L.) Merr. to rats induced CCl₄ toward hepatic IL-6 expression and ALT-AST level

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Abstract. Carbon tetrachloride (CCl₄) is one of the hepatotoxic compounds that can cause hepatic fibrosis. Fermented soybean (*Glycine max* (L.) Merr.) has higher isoflavone and fibrinolytic enzymes that could degrade fibrin substrates. The aim of this research was to know the effect of fermented soybean (*Glycine max* (L.) Merr.) to IL-6 expression and ALT-AST level in rats (*Rattus norvegicus*) that induced by CCl₄. The groups consist of negative control group, positive control group, 3 treatment groups which used dose 200, 400, 800 mg/kg BW. The treatments were conducted 2 mL/day along 14 days. Rats were euthanized then liver and serum were collected. The IL-6 expression of liver was measured by Immunohistochemistry and AST-ALT level used spectrophotometer. The parameters were analyzed statistically quantitatively using one-way ANOVA and continued test of Post hoc Tukey (α = 0.05). The result showed that dose of 800 mg/kg BW was an effective dose which has the highest percentage reduction both of hepatic IL-6 expression (68,10%) and ALT-AST level 81,16% and 46,45% respectively. The effect was related isoflavone from fermented soybean that acted as anti-inflammatory. The conclusion was the fermented of soybean able to decrease the hepatic IL-6 expression and ALT-AST level in induced CCl₄ rats.

1. Introduction
Failure of liver regeneration could induce forming of fibrotic tissue [1]. It would be become liver fibrosis if not be treated soon. Most cause to inhibition of hepatocyte proliferation is toxin such as Carbon Tetrachloride (CCl₄) which CCl₄ has high activity to induce necrosis to hepatocyte following acute liver inflammation. [2]. Occurring necrosis in hepatocyte will release Aspartate Transaminase (AST) and Alanine Aminotransferase (ALT) into the bloodstream [3]. If the liver regeneration is inhibited, hepatic stellate cell could be fibroblast and secreted extracellular matrix (ECM) to being fibrotic tissue [4]. One of cytokine proinflammatory regulate hepatocyte and fibroblast proliferation is Interleukin-6 (IL-6) [5].

Fermented food products have been known that contained fibrinolytic enzyme [6]. Fermented soybean as known as “Tempe” in Indonesia is very famous food for people [7]. Fermented soybean was reported that containing fibrinolytic enzyme as called as serine protease [8]. Serine protease has ability to degrade fibrin and extracellular matrix (ECM) [9]. Therefore, the hepatocyte regenerates the liver tissue. Soybean (*Glycine max* (L.) Merr) contains isoflavone as good antioxidant and has anti-inflammatory act. Isoflavone can inhibit inducible Nitric Oxide Synthase (iNOS) and depress NF-κB transcription factor so that cytokine proinflammatory are not be synthesis and secreted from inflammatory cells. Therefore, inflammation could be reduced [10]. The objectives of the research was...
to examine the effect of fermented soybean toward hepatic IL-6 expression and AST-ALT level in induced CCl₄ rats.

2. Materials and methods

2.1. Groups
As many as 20 rats Wistar, male, 150-200 gram BW were divided to 5 groups. Those groups were 1) negative control; 2) positive control (induced CCl₄); 3) treatment group I (induced CCl₄ + fermented soybean dose 200 mg/kg BW); 4) treatment group II (induced CCl₄ + fermented soybean dose 400 mg/kg BW); 5) treatment group III (induced CCl₄ + fermented soybean dose 800 mg/kg BW). The ethical clearance for animal using of this research was approved by Ethical Committee Brawijaya University with number certificate 745-KEP-UB.

2.2. Induction of CCl₄
CCl₄ was administrated 20% (w/v) intraperitoneally with a volume of 0.2 mL / 100g BW two times per week for two weeks. Then, The administration became twice per week for the following four weeks [11].

2.3. Preparation of fermented soybean
A total of 500 grams of soybean seeds were washed with aquadest, then boiled for 15 minutes. After washed, the soybean seed were peel and cut into smaller sizes, soaked in aquadest (1: 3, w/v) for 24 hours. After soaked, it was washed and boiled again with aquadest (1: 4, w/v) for 15 minutes and drained for 30 minutes. The fermentation of black soybean seeds by Rhizopus oligosporus was carried out in a sterile petri dish. Spore suspension of Rhizopus oligosporus was prepared by adding 10 mL of sterile 0.9% NaCl to the inoculum tube that had been incubated for 72 hours, vortexed until the fungal spores were released from the medium (± 15 minutes). Each petri dish contained 100 grams of seeds and fermented with 10 mL spore suspension and mixed until homogeneous. All petri dishes were incubated at 30±2 °C for 43 hours [7].

2.4. Administration fermented soybean
Fermented soybean was weighed as a dose based on treatment groups and then diluted in 2 mL aquadest per rat. The administration was conducted orally during 14 days.

2.5. Sample collection
In the 15th days, rats were euthanized and liver and blood was collected. Liver were immersed in formaldehyde 4% and blood was centrifuged to being serum. Liver were continued to tissue slide processing with paraffin method.

2.6. Examination of Hepatic IL-6 expression
Hepatic tissue slide was stained with Immunohistochemistry (IHC) for IL-6 detection. Primary antibody was mouse Ab anti IL-6 rat. The IHC staining used IHC kit Scytek for Secondary Antibody Polyvalent Biotinylated, Streptavidin Horse radish peroxidase and Diaminobenzidine and H₂O₂. Counter staining used Mayer Hematoxylin. Hepatic IL-6 expression was measured using software Image J with ImmunoRatio plugin. ImmunoRatio could be quantitatively measurement for IHC picture as percentage of expression area. Every IHC slides were captured 5 pictures (top-left, top-right, middle part, bottom left and bottom right). The area expression was averaged to being representative of the hepatic IL-6 expression of each rat.

2.7. Examination of AST-ALT level
AST and ALT was measured in serum using AST and ALT reagent kit. AST reagent was consisting of reagent I: TRIS, L-aspartate, malate dehydrogenase, lactate dehydrogenase and reagent II: 2-oxaloglutarate, NADH. ALT reagent was consisting of reagent I: TRISS, L-alanine, Lactate dehydrogenase and Reagent II: 2-oxaloglutarate, NADH. Measurement AST and ALT at serum was conducted by spectrophotometer at a wavelength 365 nm to get Optical Density (OD) after mixed with reagent respectively. OD readings were repeated 3 times at 1-minute intervals. Delta absorbent / minute was then multiplied by the factor to get AST and ALT level (U/L). Factor for AST and ALT are 1746 and 1768 respectively.

2.8. Data analysis
Quantitative data including IL-6 expression and AST, ALT level was analyzed statistically with One Way ANOVA and continued with Tukey Test (p<0.05) comparing among groups.

3. Results

3.1. Hepatic IL-6 expression
CCl₄ induction showed that it could increase significantly (p<0.05) the expression of cytokine proinflammatory such as IL-6. The result of the research showed that the higher dose of fermented soybean can make lower hepatic IL-6 expression (table 1). Although the significant reduction (p<0.05) were which treated by fermented soybean at dose 400 mg/kg BW and 800 mg/kg and no significantly difference (p≥0.05) between positive control group and dose 200 mg/kg BW. The best reduction of hepatic IL-6 expression (68.10%) was looked in 3rd treatment groups which given fermented soybean 800 mg/kg BW. The expression was looked in macrophage and hepatocyte (figure 1A-E).

| Groups                      | Means of IL-6 area expression (%) | Hepatic IL-6 expression (%) |
|-----------------------------|-----------------------------------|-----------------------------|
| Elevation                   | Reduction                         |                             |
| Negative control            | 10.85 ± 1.53a                      | 80.49                       |
| Positive control            | 55.62 ± 3.48c                      | 12.26                       |
| Fermented Soybean 200 mg/kgBW | 48.80 ± 4.49c                      | 41.71                       |
| Fermented Soybean 400 mg/kgBW | 32.42 ± 4.26b                      |                             |
| Fermented Soybean 800 mg/kgBW | 17.72 ± 2.85a                      | 68.10                       |

Notes: Different superscript notation indicated has significant difference

3.2. AST-ALT level
CCl₄ caused to increase the AST and ALT level and has the highest level (p<0.05). The treatment of fermented soyben for induced CCl₄ rats decreased AST and ALT level. The lowest AST and ALT level among treatment group has been by dose 800 mg/kg BW (p<0.05) with 46.45% and 81.16% respectively (table 2 and 3), and ALT level could become similar with negative control (p>0.05).
Figure 1. The hepatic IL-6 expression. A. Negative control group; B. Positive control group (induced CCl4); C. Treatment group I (fermented soybean 200 mg/kg BW); D. Treatment group II (fermented soybean 400 mg/kg BW); E. Treatment group II (fermented soybean 800 mg/kg BW). Result showed that the reduction of IL-6 expression occurred following the elevation of dose fermented soybean which looked by the yellowish chocolate decrease in treatment group but looked a lot in positive control. (IL-6 IHC, 40x objective magnification).

Table 2. Means of AST level among the groups

| Groups                      | AST blood level (U/L) | AST blood level % |
|-----------------------------|-----------------------|-------------------|
|                             | Elevation             | Reduction         |
| Negative control            | 55.75 ± 6.85⁹         |                   |
| Positive control            | 271.75 ± 26.29⁹       | 79.48             |
| Fermented Soybean 200 mg/kgBW | 195.25 ± 7.93⁹       | 28.15             |
| Fermented Soybean 400 mg/kgBW | 178.75 ± 9.88⁹       | 34.22             |
| Fermented Soybean 800 mg/kgBW | 145.50 ± 11.50⁹     | 46.45             |

Notes: Different superscript notation indicated has significant difference
### Table 3 Means of ALT level among the groups

| Groups                      | ALT blood level (U/L) | ALT blood level % |
|-----------------------------|-----------------------|-------------------|
|                            | Elevation             | Reduction         |
| Negative control            | 26.00 ± 4.97<sup>a</sup> |                   |                   |
| Positive control            | 176.50 ± 14.39<sup>d</sup> | 85.26             |
| Fermented Soybean 200 mg/kgBW | 95.00 ± 9.49<sup>c</sup> | 46.17             |
| Fermented Soybean 400 mg/kgBW | 71.25 ± 7.93<sup>b</sup> | 59.63             |
| Fermented Soybean 800 mg/kgBW | 33.25 ± 2.22<sup>a</sup> | 81.16             |

Notes: Different superscript notation indicated has significant difference.

### 4. Discussion

Induction of CCl<sub>4</sub> was proven to make liver inflammation. The result showed that CCl<sub>4</sub> induction group has the highest IL-6 expression and AST-ALT level. Mechanism CCl<sub>4</sub> to induce liver inflammation reported that it could influence genes transcription and protein synthesis [12]. CCl<sub>4</sub> works not only directly make hepatocyte necrosis in oxidative stress pathway, but also induce protein matrix secretion, and inhibit pathway to hepatocyte genes related proliferation. Therefore, CCl<sub>4</sub> is toxin which used to hepatic fibrosis animal model.

Result of research showed that fermented soybean work in two ways to liver of induced CCl<sub>4</sub> rats. First ways are fermented soybean to be acted as anti-inflammatory effect that can reduce the hepatic IL-6 expression. Isoflavone substance of soybean worked to inhibit iNOS. The inhibition of iNOS effected to depress transcription factor NFκβ and then macrophage can not to synthesize cytokine pro inflammatory such as IL-6 [10]. However IL-6 could depress hepatocyte regeneration and induce collagen synthesis therefore it make fibrotic tissue in liver [5]. Hepatic stellate cells could produce cytokine IL-6 [13]. Isoflavone from fermented soybean acted to decrease IL-6 expression in liver.

The second way of fermented soybean in induced CCl<sub>4</sub> rats was repairing liver tissue showed from reduction significant of AST-ALT level. The act was related with fibrinolytic enzyme which contained in fermented products. The enzyme of fermented soybean reported as serine protease [8]. Serine protease also reported found in fungal fermentation [9]. Serine protease worked to degrade fibrin and collagen with breakdown peptide bond in protein [14], therefore hepatocyte could proliferation to fulfil necrotic area. The liver tissue would be back a normal and ALT level becoming normal also. The unique result was AST showed reduction but not back a normal level. It was suspected that there has disturbance metabolism pathway inside hepatocyte due to effect of CCl<sub>4</sub>. However, fermented soybean has promising effect to repair liver tissue due to toxin influence.

### 5. Conclusion

The conclusion of the research was the fermented soybean (Glycine max (L.) Merr.) could able to reduce the hepatic IL-6 expression, and lowering of AST-ALT level in induced CCl<sub>4</sub> rats.

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