

# The Effect of Antioxidant Compounds in Ethanol Extract *Inocarpus Fagiferus* Fosb Seed to Expression TNF- $\alpha$ And IL-6 Liver Cells In Hypercholesterolemia Wistar Rat

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## Abstract

*Inocarpus fagiferus* Fosb seed is empirically has an ability to reduce oxidative stress diseases such as atherosclerosis and inflammatory. The antioxidant compounds such as linoleic acid, ethyl linoleic, ethyl oleic, and homopterocarpine in ethanol extract of gayam seed (*Inocarpus fagiferus* Fosb) proved to prevent inflammation through decrease TNF- $\alpha$  and IL-6 expression liver cell of Wistar rat with high cholesterol diet for 16 weeks. This was an experimental study with randomized posttest only control group design. The samples were 25 Wistar rat, randomized into 5 treatment groups, i.e. group P<sub>0</sub> (negative control), group P<sub>1</sub> (positive control, feed high cholesterol diet), and treatment groups P<sub>2</sub>, P<sub>3</sub>, P<sub>4</sub> (high cholesterol diet and ethanol extract in dose of 50, 100, and 150 mg/kg bw respectively). After 16 weeks treatment all rats were then euthanasia to obtain their liver for immunohistochemistry analyzed to give expression of TNF- $\alpha$  and IL-6 data. All of data was analyzed by Anova to obtain the treatment different toward control by statistically with significance at  $\alpha = 0.05$ . The results showed that ethanol extracts of *Inocarpus fagiferus* Fosb seed in doses of 50-100 mg/kg bw decrease the expression of positive TNF- $\alpha$  significantly ( $p < 0.05$ ), but not significantly ( $p > 0.05$ ) for the expression of positive IL-6 liver cell hypercholesterolemia wistar rat.

**Keywords:** [*Inocarpus fagiferus* Fosb, antioxidants, antiinflammatory, TNF- $\alpha$ , IL-6, atherosclerosis]

## INTRODUCTION

*Inocarpus fagiferus* Fosb or in Bali known as gatep is one of the herbs, whose seeds are ethnobotanically be used for anti-inflammatory and antioxidant, and have various medicinal properties (Heyne, 1987; Segatri, 1995; Pauku, 2006). The ethanol extract of *Inocarpus fagiferus* Fosb seed contains unsaturated fatty acid or esters of unsaturated fatty acids such as linoleic acid, ethyl linoleic, ethyl oleic, and homopterocarpine. These compounds evidenced can increase activity of antioxidant endogen SOD and decrease the levels of lipid profile i.e total cholesterol, triglyceride, and LDL cholesterol, except the HDL cholesterol in blood plasm of Wistar rat so it are potential to prevent atherosclerosis disease (Sukadana *et al.*, 2015). Futhermore, the study showed that ethanol extracts of *Inocarpus fagiferus* Fosb seed able to induce endogenous antioxidant SOD in doses of 50-150 mg/kg bw by increasing the expression of positive SOD-3 aorta endothel cell and decrease the concentration of MDA significantly ( $p < 0.05$ ), were biomarker antioxidant and antiatherosclerosis (Sukadana and Santi, 2015).

The ability of ethanol extract *Inocarpus fagiferus* Fosb seed to induce endogenous antioxidants and prevents oxidation of LDL-cholest depends on the number of SOD in the body that are expressed in a cell. If the body have less the number of SOD so the body requires consumed exogenous antioxidant that can source of nature. Morethan antioxidant activity, these extract expected to antiinflammatory to prevent damage liver cells that was caused by stress oxidative hypercholesterolemia through to decrease TNF- $\alpha$  and IL-6 expression.

Therefore the ethanol extract of *Inocarpus fagiferus* Fosb seed can prevent to atherosclerosis through

antioxidant mechanism so it is crucial to investigate their effect toward expression of TNF- $\alpha$  and IL-6 as an antiinflammatory agent to prevent damage liver cell. In this paper the expression of TNF- $\alpha$  and IL-6 in liver cell are described.

## MATERIALS AND METHODS

### Materials

Materials used in this research are: gayam seed (*Inocarpus fagiferus* Fosb) seed, obtained from Tabanan Bali and taxonomically identified by LIPI's Kebun Raya "Eka Karya" Bali. Animal used is Wistar rat and with ethical clearance No. 0144/KE-PH/IX/2013 dated: 4 September 2013 as previously reported (Sukadana and Santi, 2015). Chemicals used are ethanol (p.a and technical) and aquadest. Chemicals that used to immunohistochemical analysis are formalin 10%, buffered citrat 0,1 M (pH. 7,4), kit LSAB (Dako, Denmark) and antibody primer *Rabbit Anti-TNF- $\alpha$  Polyclonal Antibody* (Bioss, Cat. bs-3895R), *Rabbit Anti-IL-6 Polyclonal Antibody* (Bioss, Cat. bs-0379R) and another chemicals from Sigma-Aldrich (USA) as metanol p.a, thripsine 0,25%, H<sub>2</sub>O<sub>2</sub> 3%, formalin buffered phosphate 10%, alcohol 70%, 90%, 96%, 100%, xylene, paraphine FBS 5%, *Biotinylated Goat Anti-Polyvalent, Streptavidin peroxidase*, aquabidest, hematoxylin Gill, and buffer tri sodium citrat. Other material were Whatman No.1, ketamine and xylazine for anesthesia.

### Equipments

Equipments used include a set of glass wares, microtom rotary (Jung Histocut Leica 820), microscope binocular Olympus CX41 with camera Olympus DP12, photographed with an Optilab Pro (Miconos, Indonesia)

camera. Each preparation was photographed 5 times by in JPEG format using Optilab Raster Image Viewer 1.0 and 2.1 software (Miconos, Indonesia). microscope slide polylysine, and coverslip.

#### Procedure Extraction of Gayam Seed and Apply to Wistar Rat

Protocol of extraction and application to wistar rat as previously reported. To prove the potency of concentrated EtOH extract of *Inocarpus fagiferus* Fosb seed to antiinflammatory agent through decrease of expression TNF- $\alpha$  and IL-6 liver cell using to randomized posttest only control group design with 5 groups.

Group P<sub>0</sub>: negative control (rat group with feed standard)

Group P<sub>1</sub>: positive control (rat group with feed high fat diet)

Group P<sub>2</sub>: rat group with feed high fat diet + ethanol extract dose 50 mg/kg bw

Group P<sub>3</sub>: rat group with feed high fat diet + ethanol extract dose 100 mg/kg bw

Group P<sub>4</sub>: rat group with feed high fat diet + ethanol extract dose 150 mg/kg bw.

The experiment was conducted for four months then liver cell of each rat groups were analyzed for their expression TNF- $\alpha$  and IL-6. These data were analysed using one way Anova (SPSS version 19.0 for Windows). Descriptive analysis to provide an overview of the characteristics data. Test for normality with Shapiro-Wilk test, and homogeneity with Levene Test. Comparison test conducted by One Way Anova and to see the difference between groups was followed by Post Hoc Test LSD. All tests with significance level of 5% ( $\alpha = 0.05$ ).

### RESULTS AND DISCUSSION

#### Expression of TNF- $\alpha$ Liver Cell

Figures 1 and 2 describe the average and an overview of positive expression of TNF- $\alpha$  rat liver cells based on immunohistochemical analysis using Rabbit Anti-TNF- $\alpha$  polyclonal antibody cell marked brown on the edges or the cell nucleus, whereas TNF- $\alpha$  expression negatively characterized by liver cells are colored blue on the edges or the cell nucleus.

In the group of rat fed with high cholesterol (P<sub>1</sub>) caused to accumulate of intracellular lipids. This accumulation will increase the demand on the endoplasmic reticulum (ER), which integrates many metabolic processes. Endoplasmic reticulum dysfunction leads to production of reactive oxygen species (ROS) it provoking oxidative stress and then activation of TNF- $\alpha$  inflammatory pathways. Oxidative stress can also induce DNA damage that leads to genomic instability. Moreover, the adipose tissue is increasingly viewed not simply as a reservoir of stored energy, but rather as an active secretory organ (Zhang and Kaufman, 2008).

As shown in Figures 1 and 2, the groups of rat in P<sub>4</sub> have not significantly higher expression of TNF- $\alpha$  positive ( $p > 0.05$ ) when compared with positive controls P<sub>1</sub>, while the groups of rat in P<sub>2</sub> and P<sub>3</sub> have significantly lower expression ( $p < 0.05$ ). These mean the ethanol extract of *Inocarpus fagiferus* Fosb seed in doses of 50-100 mg/kg bw able to prevent inflammation of liver cell but in doses of 150 mg/kg bw shown increasing of expression, which means in these doses it causes liver damage. The decreases in the expression of TNF- $\alpha$  positive in P<sub>2</sub> and P<sub>3</sub> groups are due to the antioxidant compounds contained in the ethanol extract of gayam seeds such as linoleic acid, ethyl linoleic, ethyl oleic, and homopterocarpine (Sukadana *et al.*, 2015) which can inhibit the oxidation reaction by the way it reacts with superoxide anion ( $\cdot O_2^-$ ) to form relatively stable molecules. Decrease in the number of superoxide anion radicals prevent new free radicals formation, and the formation of molecules which are relatively more stable, which play an important role in antiinflammatory agent. Tumor necrosis factor (TNF- $\alpha$ ) known as a pleiotropic cytokine which it play an important role in dicotomy in the liver cell i.e; as a mediator cell damage and induce to proliferation as like hepatocyte and regeneration liver cell (Schwabe and Brenner, 2006).

#### Expression of IL-6 Liver Cell

The average expression of IL-6 positive in all groups that were immunohistochemical analysis described in Figure 3 and 4.

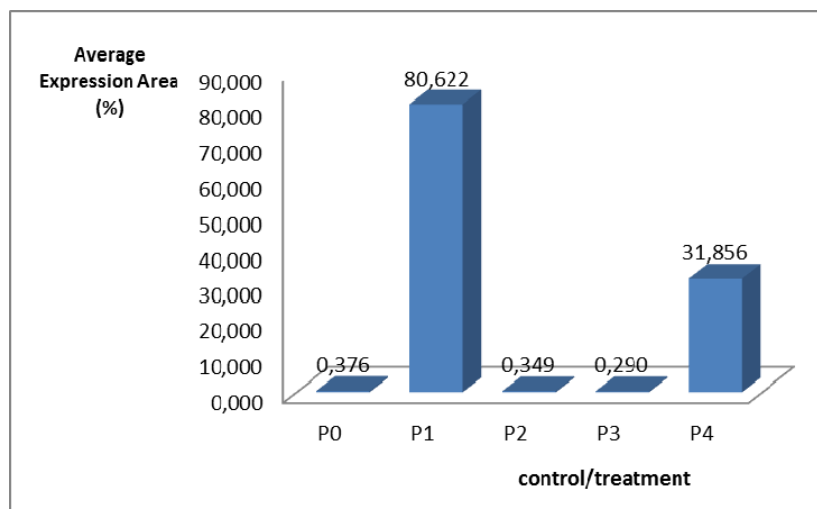
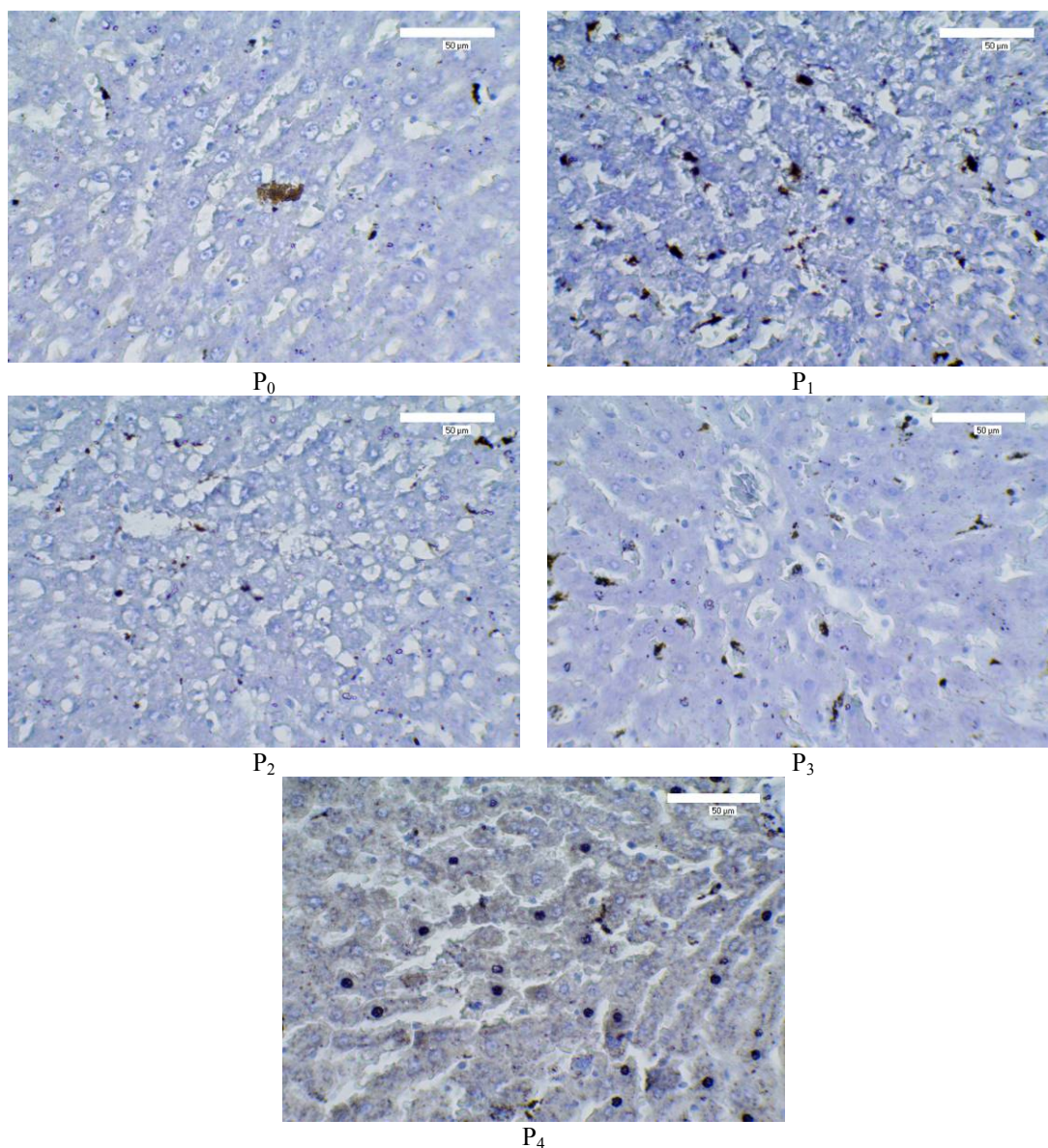


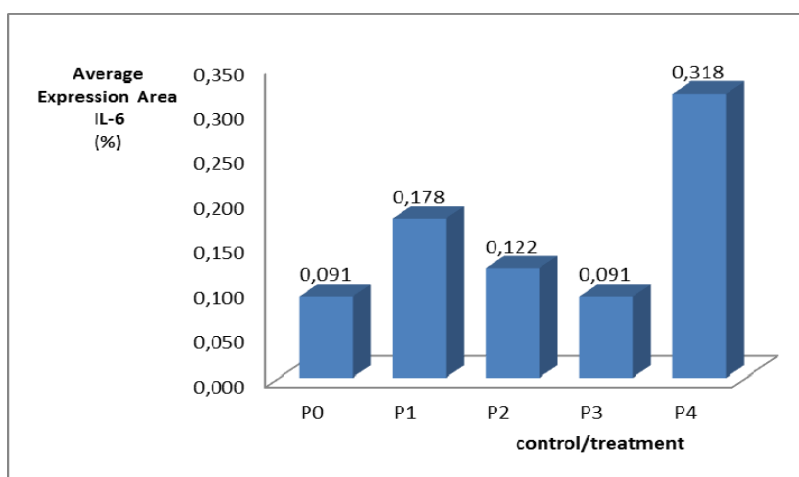
Figure 1.

The average expression of TNF- $\alpha$  Positive in all groups. The result of the average difference between groups: P<sub>0</sub> vs. P<sub>1</sub>,  $p < 0.05$ ; P<sub>2</sub> vs P<sub>1</sub>,  $p < 0.05$ ; P<sub>3</sub> vs P<sub>1</sub>,  $p < 0.05$ ; P<sub>4</sub> vs P<sub>1</sub>,  $p > 0.05$ ; P<sub>2</sub> vs P<sub>3</sub>,  $p > 0.05$ ; P<sub>2</sub> vs P<sub>4</sub>,  $p > 0.05$ ; P<sub>3</sub> vs P<sub>4</sub>,  $p > 0.05$ .



**Figure 2.**

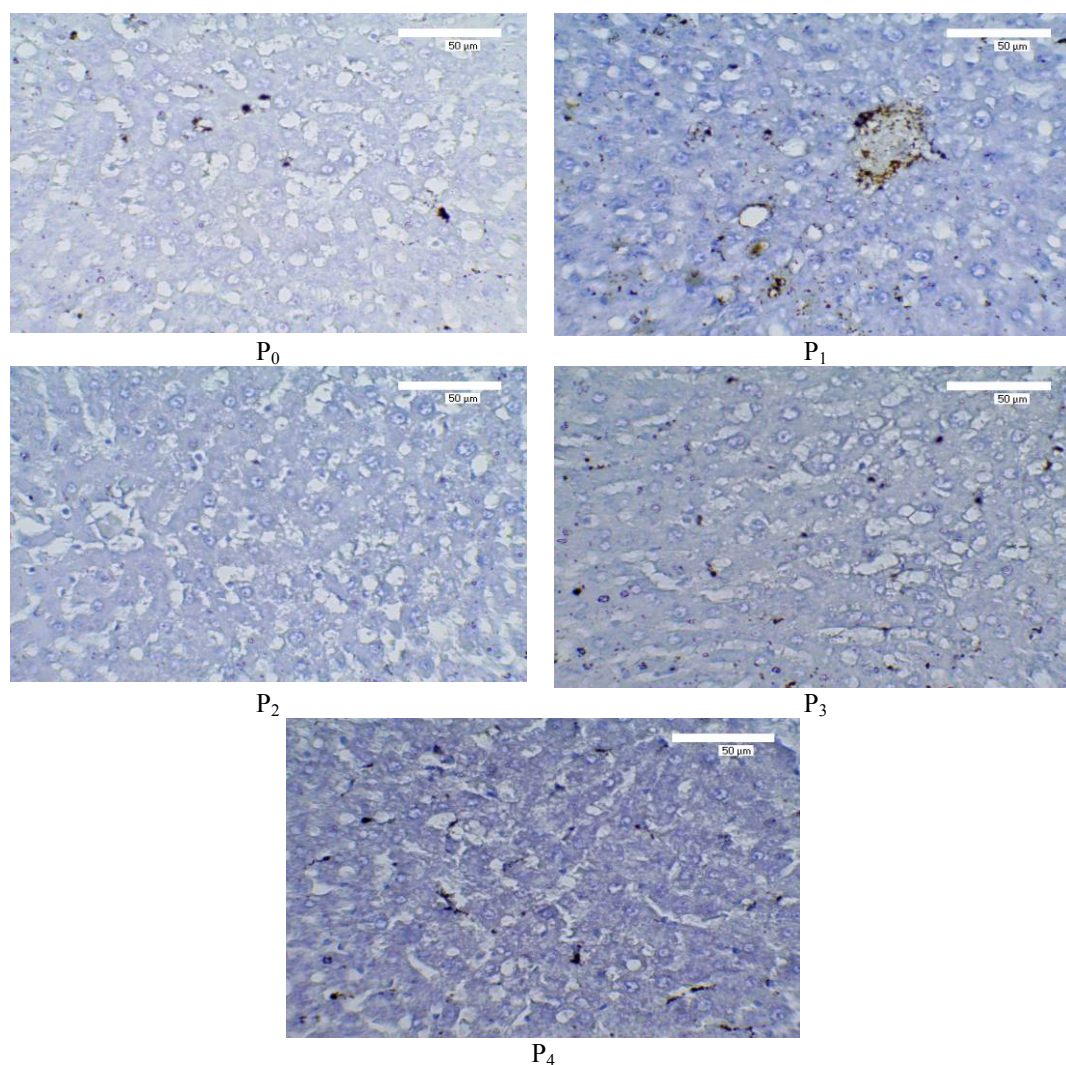
TNF- $\alpha$  Expression Liver Cell Wistar rat Hypercholesterolemia Control and Treatment Groups (P<sub>0</sub>,P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub>, and P<sub>4</sub>)



**Figure 3.**

The average expression of IL-6 Positive in all groups. The result of the average difference between groups: P<sub>0</sub> vs. P<sub>1</sub>,  $p > 0.05$ ; P<sub>2</sub> vs P<sub>1</sub>,  $p > 0.05$ ; P<sub>3</sub> vs P<sub>1</sub>,  $p > 0.05$ ; P<sub>4</sub> vs P<sub>1</sub>,  $p > 0.05$ ; P<sub>2</sub> vs P<sub>3</sub>,  $p > 0.05$ ; P<sub>2</sub> vs P<sub>4</sub>,  $p < 0.05$ ; P<sub>3</sub> vs P<sub>4</sub>,  $p < 0.05$ .





**Figure 4**

IL-6 Expression Liver Cell Wistar rat Hypercholesterolemia Control and Treatment Groups (P<sub>0</sub>, P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub>, and P<sub>4</sub>)

As like in sitokin inflammatory TNF- $\alpha$ , the high intake of cholesterol (hypercholesterolemia) increased expression IL-6 liver cell not significantly in group P<sub>1</sub> compared to standard groups (P<sub>0</sub>) ( $p > 0.05$ ), and P<sub>2</sub>, P<sub>3</sub>, and P<sub>4</sub> ( $p > 0.05$ ) toward P<sub>1</sub>, as shown in Figure 4. Hypercholesterolemia conditions as in P<sub>1</sub> will increase intracellular lipids accumulation. This lipids accumulation will increases demand on the endoplasmic reticulum (ER) which is leading to uncontrolled production of reactive oxygen species (ROS). ROS then stimulate inflammatory signaling and induce oxidative damage, including strand breaks and nucleotide modifications (Zhang and Kaufman, 2008). Therefore the expression of IL-6 in group P<sub>1</sub> to be increased. In a recent issue shown that hypercholesterolemia induces production of IL-6 and TNF- $\alpha$  cytokines, which are required for the initiation and progression of hepatocellular carcinoma (HCC). IL-6 ad TNF- $\alpha$  activate STAT3 and NF- $\kappa$ B, respectively, which promotes cell proliferation of damaged cells through transcriptional alterations and pathway activation in damaged hepatocytes contribute to HCC development (Park et al., 2010).

The groups of rat in P<sub>2</sub> and P<sub>3</sub> have not significantly lower expression ( $p > 0.05$ ) toward P<sub>1</sub>. These mean the ethanol extract of *Inocarpus fagiferus* Fosb seed in doses of 50-100 mg/kg bw able to prevent inflammation of liver cell but in doses of 150 mg/kg bw shown increasing of expression, which means in these doses it causes liver damage. The decreases in the expression of TNF- $\alpha$  positive in P<sub>2</sub> and P<sub>3</sub> groups are due to the antioxidant compounds contained in the ethanol extract of gayam seeds.

#### CONCLUSION

Conclusions of these researches are:

1. Ethanol (EtOH) extract of gayam seed that contains antioxidant compounds in doses of 50-100 mg/kg bw can prevent to inflamamtion by decreasing the expression of TNF- $\alpha$  liver cell in hypercholesterolemia Wistar rat significantly.
2. Ethanol (EtOH) extract of gayam seed that contains antioxidant compounds in doses of 50-100 mg/kg bw can prevent to inflamamtion by decreasing the expression of IL-6 liver cell in hypercholesterolemia Wistar rat not significantly.

#### FURTHER RESEARCH

In vivo experiment of ethanol extract to obtain its potential as antiinflamamtory with relevant variables.

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