Hepatoprotective Effect of Ganoderma applanatum Crude Polysaccharides on Carbon Tetrachloride-Induced Early Liver Fibrosis in Mice

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ABSTRACT

Introduction: Liver fibrosis is a treatable disease which still at early stages. Ganoderma applanatum plays a role as alternative medicine. The fungi have antioxidant, anti-inflammatory, and anticancer bioactivities. This study aimed to evaluate the hepatoprotective effects of G. applanatum crude polysaccharides (GACP) on liver fibrosis due to CCl₄ induction. Methods: This study was divided into six groups. BALB/c mice were given CCl₄ dissolved in olive oil (2 mL/kg: 1:3) intraperitoneally (i.p) twice a week for four weeks to produce a liver fibrosis model. Distilled water (control group), silymarin 100 mg/kg and GACP 25, 50, 100 mg/kg were given once daily for four weeks. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) were determined by enzyme-linked immunosorbent assay (ELISA) kit. Histopathology was stained by hematoxylin & eosin (H&E) and Masson’s trichrome. Results: The administration of GACP effectively prevented ALT, AST, TNF-α, and IL-6 levels from high elevation. Additionally, the GACP had protective effect after liver histological analysis exhibited less injury in the liver tissue. Conclusion: The hepatoprotective effect of GACP on liver fibrosis is mainly due to anti-inflammatory, Carbon tetrachloride, Crude polysaccharides, Fibrosis, Ganoderma applanatum.

INTRODUCTION

The liver is known as central metabolism and detoxification for all type of hepatotoxic agents. Many factors can cause liver damage, such as viral infections, autoimmune diseases, and xenobiotic hepatotoxicity. Liver fibrosis is a wound healing process to reverse the liver injury chronic stage which is characterized by excess extracellular matrix due to liver inflammation. Fibrosis is generally associated with an unhealthy life such as alcohol consumption, obesity, genetic, hepatotoxicant, and hepatitis infections. Finally, liver fibrosis leads to more severe stages such as cirrhosis and hepatocellular carcinoma. Carbon tetrachloride (CCl₄) is an environmental toxicant which potential to induce liver damage by increasing reactive free radicals. CCl₄ metabolism by cytochrome P450 in endoplasmic reticulum affect an increase in trichloromethyl radicals (CCl₃•) which play role in liver toxicity. Various types of P450 are present in the liver endoplasmic reticulum, one of them is cytochrome P450 2E1 (CYP2E1) which is involved in metabolic processes and metabolic enzymes activation when liver injury by CCl₄ induction. The pathogenicity of CCl₄ in inducing liver damage includes many factors such as inflammation, oxidative stress, and apoptotic reactions. These CCl₄• bind nucleic acids, proteins, and lipids whereas these actions impair liver tissue via mutation, decreasing protein synthesis, and lipid peroxidation. All of injury pathways are end in hepatic toxicity. Subsequently, this phase leads to inflammation pathway which increase several pro-inflammatory cytokines such as IL-6 and TNF-α. Furthermore, hepatic stellate cells (HSCs) are quiescent cells that can be activated into myofibroblasts when they receives a stimulation from pro-inflammatory cytokines and growth factors. Thus, it plays an important role in chronic liver injury progression. Moreover, this process leads to produce additional extracellular matrix (ECM) components and into fibrosis stage. HSC activation is directly involved with increased collagen density. In particular, TNF-α and IL-6 stimulate HSCs into myofibroblast stage. This transdifferentiation stage plays major role in producing ECM in wound healing process. Hence, the myofibroblast is key factor of fibrosis. Although, there have been significant advances in information regarding the molecular pathology of liver injury, there is still no alternative treatment that effectively prevents or treats liver fibrosis. Silybum marianum (silymarin) has long been known as a liver therapy natural drug with high antioxidant and anti-inflammatory activities. Recently, extracts of natural ingredients are often used widely for the prevention of diseases in animals. Many studies have reported on natural antioxidants from polysaccharides, ketones, alkalis or glycosides in inhibiting reactive oxygen species (ROS) that induce liver damage. Polysaccharides are often a positive effect when used as herbal therapy against liver injury. Furthermore, they are also easily found in animals, plants and microbes. Ganoderma is one of the medicinal mushroom groups and being hepatoprotector agent with no side effects. The effectiveness of fungi absorption in the body through...
clathrin and caveola mediated endocytosis route by micropinocytosis which is then absorbed by epithelial cells and circulated into the blood.13-20 *Ganoderma* polysaccharides have been proposed as bioactive components to prevent liver injury due to toxic substances. Previous studies have shown that polysaccharides have immunomodulatory, anticancer, anti-aging, anti-diabetic, and liver protection activities. However, there is still missing information regarding possible mechanisms of liver protection. This study was designed to evaluate the hepatoprotective effects of GACP against CCl₄ inducing liver fibrosis in mice.

**MATERIALS AND METHODS**

**Extract preparation**

*G. applanatum* was obtained from Tulungagung, East Java, Indonesia. Crude polysaccharides were generated from *G. applanatum* basidiocarp. The 400 g of *G. applanatum* dry powder dissolved in 4 L water to a boil at 75°C for two times (three hours each). The insoluble portion was removed during filtration, and the supernatant was centrifuged (2000×g; 10 minutes) and filtered again. The supernatant was collected and precipitated with absolute ethanol (1:4) three times. The precipitate was then dissolved into water and lyophilized.27

**Acute toxicity test**

The results of acute toxicity test were used to determine GACP extracts toxicities whether showed adverse effects or not. This test based on Organization for Economic Cooperation and Development (OECD) guidelines, 2002. Mice were divided into several groups with six mice of each group and administered with distilled water; and increasing dose start from 25 mg/kg, 50 mg/kg, 100 mg/kg, 200 mg/kg, 400 mg/kg, 800 mg/kg, and 1600 mg/kg body weight. The mice were kept in fast overnight but was still got free access water before treatment. The mice were also fed on 4 hours after dosing and were evaluated for 30 min and 2, 3, 4, 24 hours after dosing to progress information about any sign toxicity and clinical or toxicological symptoms.

**Experimental animal preparation and drug administration**

A total of 24 mice (4 weeks old, weighing 30-35 g) were used. Room temperature was kept at 25±1°C, and relative humidity at 60±5%. Mice were subjected to a 12 hours day/night cycle (lights turn on at 7 am and turn off at 8 pm). All mice were acclimatized to laboratory conditions for one week prior to testing. During the test, the mice were given water and *ad libitum* feed and weighed every week to monitored their health condition. The mice were divided into six groups: normal control group; Oral distilled water (DW) and olive oil; model control group: CCl₄ (Merck, Germany) and DW oral administration; silymarin group; Oral administration with silymarin 100 mg/kg and CCl₄ low GACP; Oral administration 25 mg/kg and CCl₄ medium GACP; Oral administration 50 mg/kg and CCl₄ high GACP; Oral administration 100 mg/kg and CCl₄. Inducing hepatic fibrosis, a dose of 2 mL/kg bodyweight CCl₄ was injected intraperitoneally (i.p) twice a week for four weeks (dissolved in olive oil, 1:3). Furthermore, DW, silymarin, and GACP were given per-oral once daily every day for four weeks. On the fifth week, the animals were sacrificed and the liver was taken for weighed, histological and biochemical examination, such as levels of inflammatory indicators. Liver weight index were calculated. Data in Table 1 showed no significance (p>0.05) on week 1, week 2 and week 3 between treatment groups. However, measurement carried out during the study showed that administering CCl₄ on week 4 significantly lowering body weight (p<0.05) in comparison with normal control group and also displayed the lowest weight value (21.01 ± 3.07 g). On week 4. All of GACP groups and silymarin exhibited significantly increased (p<0.05) compare than model control group and silymarin group showed a highest value (36.07 ± 4.16 g). Furthermore, in Figure 1A, CCl₄ was also significantly increased liver weight (p<0.05) in comparison with normal control group (4.3 ± 0.32 g). Otherwise, GACP and silymarin were effectively prevented elevation of liver weight (p<0.05) after being comparison with model control group and silymarin group revealed the lowest (1.3 ± 0.08 g). Hepatic index in Figure 1B was significantly increased (p<0.05) after CCl₄ induction compare with normal control group (16.81 ± 3.12 %). However, giving GACP and silymarin were proven significantly warded off the increase in liver index (p<0.05) compared to the model control group and silymarin group exhibited the best value (3.65 ± 0.56%).

**Effect of GACP on body weight and liver index in early liver fibrosis**

To determine hepatoprotective of GACP, the body weight and liver index were calculated. As shown in Figure 2A and Figure 2B, ALT and AST levels in model control group were significantly increased (p<0.05) in comparison with normal control group and displayed the highest biomarker levels (173.5 ± 70 U/L; 484.25 ± 174.64 U/L, respectively), which indicated liver injury severity. The GACP treatment and silymarin showed significantly lower (p<0.05) ALT and AST levels compared to model control group.
Susilo RJK, et al.: Hepatoprotective Effect of *Ganoderma applanatum* Crude Polysaccharides on Carbon Tetrachloride-Induced Early Liver Fibrosis in Mice

**Table 1:** Effect of GACP on body weight in mice induced by CCl₄.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before CCl₄</td>
</tr>
<tr>
<td>Control</td>
<td>24.98 ± 7.1</td>
</tr>
<tr>
<td>Model</td>
<td>24.76 ± 3.25</td>
</tr>
<tr>
<td>GACP 25 + CCl₄</td>
<td>24.53 ± 5.44</td>
</tr>
<tr>
<td>GACP 50 + CCl₄</td>
<td>25.77 ± 6.31</td>
</tr>
<tr>
<td>GACP 100 + CCl₄</td>
<td>24.65 ± 6.8</td>
</tr>
<tr>
<td>Silymarin + CCl₄</td>
<td>24.5 ± 4.93</td>
</tr>
</tbody>
</table>

Note: GACP: *Ganoderma applanatum* crude polysaccharides. All values are the means ± SD (n = 6). *p<0.05 vs control group; **p<0.0001 vs control group; #p<0.05 vs model group.

**Figure 1:** Effect of GACP on liver weight (A) and liver index (B) in mice induced by CCl₄. Data express the mean ± SD (n = 6). *p<0.05 vs control group; ****p<0.0001 vs control group; #p<0.05 vs model group; ####p<0.0001 vs model group.

**Figure 2:** Effect of GACP on ALT (A) and AST (B) levels in mice induced by CCl₄. Data express the mean ± SD (n = 6). *p<0.05 vs control group; **p<0.0001 vs control group; #p<0.05 vs model group; #p<0.01 vs model group. ALT: Alanine transaminase; AST: Aspartate transaminase.
and GACP 100 mg/kg dose group exhibited the lowest levels value (83 ± 8.12 U/L; 150.25 ± 47.09 U/L, respectively). These things described about potential protection of GACP extracts against liver injury.

**Effect of GACP on TNF-α and IL-6 levels in early liver fibrosis**

The serum levels of pro-inflammatory cytokines such as TNF-α and IL-6 are closely related to the initiation of inflammation as a fibrosis stimulator via inflammation pathway. On Figure 3A and Figure 3B showed that model control group significantly increased (p<0.05) TNF-α and IL-6 levels compared to the normal control group and give the highest value (26.93 ± 12.28 ng/L; 4 ± 0.99 pg/mL, respectively). Meanwhile, GACP and silymarin inductions to the treatment groups effectively prevented escalation (p<0.05) of TNF-α and IL-6 levels. Furthermore, GACP 100 mg/kg dose group was the most effective dose in avoid elevation of TNF-α and IL-6 levels (5.78 ± 0.7 ng/L; 1.01 ± 0.04 pg/mL, respectively). The results displayed that GACP inhibited pro-inflammatory cytokines elevation too.

**Effect of GACP on liver histopathological in early liver fibrosis**

Histological evaluation is important to determine the degree of liver injury due to CCl₄ and effect of GACP in defending the liver tissue from that liver injury. This histological study using HE staining for identification of damage form and Masson’s trichome staining to evaluated collagen density in liver tissue. As shown in Figure 4, the normal control group showed normal structures in the liver tissue, and sinusoid and central veins were still formed normally too. In contrary, CCl₄ injection in model control group revealed necrosis, ballooning hepatocytes, inflammatory infiltration vastly in surrounding central vein. Meanwhile, the silymarin group showed prevented this injury due to CCl₄ and effect of GACP in defending the liver tissue with less side effects. In the present study, GACP medicine is still carried out to solve the problem about how to treat fibrosis effectively with less side effects. In the present study, GACP was chosen as alternative medicine showed the significant effects to attenuate fibrosis factors. Firstly, after CCl₄ induction, the weighing

**DISCUSSION**

As primary source of ECM in liver fibrosis, quiescent HSCs are transdifferentiated into myofibroblast-cell like caused by inflammation factors such as TNF-α and IL-6. Carbon tetrachloride induction as in Figure 3A and Figure 3B, showed about the normal control group with a clear hepatic structure and no collagen formation in liver tissue. Induction of CCl₄ in model control group showed little collagen fiber formation, especially in central vein area.

This formation was still not produced septa yet, but Masson’s trichome staining had already exhibited that for 4 weeks, CCl₄ injection caused this little fibrotic formation. Meanwhile, giving silymarin displayed liver amelioration with less collagen fiber which was indicated by yellow arrow. The dose 25 mg/kg of GACP revealed mild collagen fiber in central vein area compared with model control group. Moreover, GACP dose 50 mg/kg group exhibited mild collagen fiber too in central vein area. Hereafter, dose 100 mg/kg of GACP showed very delicate collagen fiber deposit in surrounding central vein area. Producing the least compared to other GACP groups and barely visible formation. Meanwhile, statistical analyzed for collagen density in Figure 5B, showed about the normal control group with a clear hepatic structure and no collagen formation in liver tissue. Induction of CCl₄ in model control group showed little collagen fiber formation, especially in central vein area.

**Figure 3:** Effect of GACP on TNF-α (A) and IL-6 (B) levels in mice induced by CCl₄. Data express the mean ± SD (n = 6). *p<0.05 vs control group; **p<0.01 vs control group; ##p<0.05 vs model group; ###p<0.01 vs model group; ####p<0.001 vs model group. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6.
**Figure 4:** Effect of GACP on liver histology in mice with H&E staining (magnification, ×200). B: Ballooning hepatocyte; CV: Central vein; I: Inflammatory infiltration; Ne: Necrosis.

**Figure 5:** Effect of GACP on liver fibrosis in mice with Masson’s trichome staining (magnification, ×200) (A). Black arrow showing the collagen area. Semi-quantitative analysis of collagen density (B). Data express the mean ± SD (n = 6). ****p<0.0001 vs control group; ##p<0.01 vs model group; ###p<0.001 vs model group; ####p<0.0001 vs model group.
displayed reducing value on every week and reach significant on week 4. Administered of GACP could elevated the weighing which is related to the role of GACP as a supplement with giving so much nutrition such as arginine, glutamic acid, aspartic acid, tryptophan, and lysine. The nutrition works in accelerate body metabolism to dispose many toxins from the body.30

Decreasing in liver weight and liver index explained that GACP play role in liver mass via decreasing collagen component which increase the weight of liver.31 In this study, the degree of liver injury was indicated by measuring the levels of liver enzyme biomarkers. CI CI induction could significantly increase ALT and AST levels compared to the normal control group. This event was occurred due to the release of these hepatic enzymes into the bloodstream after being necrosis in liver cell.32 Both of enzymes, especially, ALT is known as specific enzymes for liver injury due to their limited distribution in other organs as we know that the origin of enzyme is derived from liver cytoplasm. Meanwhile, AST is generally associated with liver and another organ injury such as the heart, muscles, kidneys, bones and brain. Increase in AST levels is indeed a benchmark of liver injury stage but no absolute due to the levels are quite a lot in other organs.33 However, AST can still be used as reference considering that its origin is fairly high in the mitochondria and cytoplasm of liver. Furthermore, treatment with GACP and silymarin showed protective activity which marked by decreasing hepatic enzymes. This indicates that GACP can trigger liver cell regeneration and reduce the leakage of hepatic enzymes in cells. In the chronic stage of damage, as injury’s response, NF-κB will activates and stimulates Kupffer cell (resident macrophage) to produce pro-inflammatory cytokines in HSCs activation.34-37

This activation aims to maintain homeostasis and also begin wound healing process whereas pro-inflammatory cytokine takes pivotal role accelerate myofibroblast-cell like transdifferentiation. TNF-α and IL-6 are pro-inflammatory cytokine that involved in fibrosis formation. Associated via inflammation pathways, TNF-α is one of the pro-inflammatory cytokines which mainly produced by macrophage. In fibrosis, this cytokine acts on myofibroblast-cell like and apoptotic activator. Moreover, IL-6 was produced by macrophage and HSCs along with hepatomitogen and also plays in accelerating transdifferentiation.38-40 Hence, inhibition of the inflammatory factor impacts to attenuate liver fibrosis.41 This study showed that CCl 4 administration significantly increased the degree of liver injury's parameters by histological evaluation. Inducing by CCl 4 process. To determine GACP effects in liver tissue, we observed liver injury’s parameters by histological evaluation. Inducing by CCl 4 causes necrosis, hydropic, ballooning hepatocytes, and inflammatory infiltration. CCl 4 toxins impair cells by homeostasis failure whereas excessive water is stuck in the cell leading to ballooning stage and ends with necrosis.42 In contrast, distinct improvement was displayed by GACP administration when could ameliorate injury parameters. As we explain in discussion above, GACP could significantly decrease pro-inflammatory factor which reduced injury response trigger to proliferate cells that repairing liver tissue.43 HSCs activation was still be used as reference considering that its origin is fairly high in the mitochondria and cytoplasm of liver. Furthermore, treatment with GACP and silymarin showed protective activity which marked by decreasing hepatic enzymes. This indicates that GACP can trigger liver cell regeneration and reduce the leakage of hepatic enzymes in cells. In the chronic stage of damage, as injury’s response, NF-κB will activates and stimulates Kupffer cell (resident macrophage) to produce pro-inflammatory cytokines in HSCs activation.34-37

Inhibiting inflammatory reactions, and decreasing collagen density in hepatic tissue. CONCLUSION

In summary, GACP showed hepatoprotective potential against early liver fibrosis through prevent pro-inflammatory cytokines elevation and collagen density. These results indicate that GACP effectively as a protector against liver fibrosis. Further clinical trial research should be conducted and fibrosis therapy in human should be with new alternative treatment methods.

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REFERENCES

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**GRAPHICAL ABSTRACT**

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