Original Article

Effects of Genistein on Altering Oxidative Stress in Rats with Alcohol-Induced Liver Injury

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ABSTRACT

BacKground: Illness and death from liver disease throughout the world is caused long term alcohol consumption. There are still no therapeutic modalities to stop or reverse the pathogenesis and progression of alcoholic liver disease. Genistein, a soy isoflavone, might provide an alternative approach to prevent or treat this disease.

Objective: To assess the effects of genistein on altering oxidative stress level in rats with alcohol-induced liver injury.

Method: Male Sprague-Dawley rats were divided into three experimental groups of six rats per group. Control group was fed distilled water (8 mL/Kg BW/day), alcohol group was fed 50% alcohol (8 g/Kg BW/day) and genistein group was fed genistein (16 mg/Kg BW/day) dissolved in 50% alcohol (8 g/Kg BW/day). All groups were treated via a gavage tube twice a day for 4 weeks. At the end of the study, all rats were sacrificed. Serum liver enzymes including serum aspartate transaminase (AST) and serum alanine transaminase (ALT), hepatic malondialdehyde (MDA) and glutathione (GSH), liver histopathology were measured.

Results: Levels of hepatic MDA and serum AST in genistein group were significantly lower than in alcohol group (0.09±0.02 vs. 0.13±0.02 and 283.33±137.92 vs. 665.17±211.35, respectively; $p<0.01$). ALT level in serum was also significantly lower in genistein group than in alcohol group (32.43±12.90 vs. 120.30±75.30; $p<0.05$). Level of hepatic GSH was significantly increased in genistein group when compared with alcohol group (14.77±2.80 vs. 11.55±1.15; $p<0.05$). The liver histopathology in alcohol group presented mild steatosis, mild lobular inflammation and mild to moderate ballooning degeneration. On the other hand, genistein group revealed the improvement of liver histopathology when compared to alcohol group.

Conclusion: The present study suggested that genistein could improve liver histopathology and liver enzymes by decreasing oxidative stress and increasing glutathione level in rats with alcohol-induced liver injury. In addition, these protective effects of genistein are possible to clinical application in patients with alcoholic liver disease.

Key words : Genistein, alcoholic liver disease, oxidative stress, glutathione

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Introduction

Alcohol (ethanol) is directly hepatotoxic. Illness and death from liver disease throughout the world is caused long term alcohol consumption[1,2]. Typically, alcohol-induced liver injury shows at the beginning as acute inflammation and next develops to steatosis (fatty liver). If alcohol intake is continued, fatty liver may advance to alcoholic hepatitis, to fibrosis, and lead to cirrhosis[3]. Alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1): especially once chronic alcohol consumption) are major enzymes to break down alcohol in the liver by changing it to a toxic substance, acetaldehyde. ADH pathway involves nicotinamide adenine dinucleotide (NAD^+) to from reduced nicotinamide adenine dinucleotide (NADH) which leads to redox state; as a result, the liver is susceptible to injure form the byproducts of alcohol metabolism including acetaldehyde and free radicals[4]. In addition, increasing NADH levels breaks down glucose production and enhances fat molecules production which promotes steatosis[5]. Acetaldehyde and reactive oxygen species (ROS), which are produced from alcohol metabolism, makes stable and unstable adducts by interaction with protein building block and other molecule[4] that causes DNA damage and lipid peroxidation in cellular membrane, and these causes interferes with physiological processes and elevates oxidative stress in the liver[6]. In general, antioxidants, especially glutathione (GSH) eliminates ROS from the cells; however, alcohol consumption decreases glutathione levels in the liver cells, especially in the mitochondria. As a result, protection mechanism of the cells on oxidative stress is impaired, and may cause cell death[5]. Furthermore, alcohol increases permeability of intestine to enhance endotoxin (negative bacteria protein) from blood steam to the liver which activates kupffer cells. Then, kupffer cells generates tumor necrosis factor alpha (TNF-alpha) which in turn activates another type of liver cell such as the stellate cells to produce scar tissue formation causing fibrosis and other chemokines (e.g., IL-8) to attract inflammatory cells inducing liver inflammation[7].

Alcoholic hepatitis patients have used steroid for several years. Nevertheless, steroid effect is limited. Because it reduces both the immune and inflammatory responses[8]. Therefore, it is still a challenge to find more effective therapy with less adverse effect for alcoholic liver injury. In addition, occurring evidence shows that the multiple mechanisms conduce to alcoholic liver disease (ALD). As a result, there is still no treatment to stop or reverse the pathogenesis of ALD. Herbal medicine is used to treat chronic diseases, including ALD, has been a common clinical practice for long time in Asian countries; because, the effect of herbal medicine is the extensive and integrated outcomes of their active components contained. Therefore, the multi-targeted herbal medicines might supply an alternative approach to protect or cure this disease[9].

Materials and Methods

Animal preparation

Male Sprague-Dawley rats, weighing about 180-220 grams, were purchased from the National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand. The animals were housed in a controlled temperature room at 25 ± 1˚C under standard conditions with 12-h light-dark cycle. All rats were received proper care in accordance with the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (approval No. 08/2556).

Genistein preparation

Genistein (Cayman Chemical Company, USA) was dissolved in 50% alcohol which freshly prepared for the experiment.

Experimental Protocols

All Male Sprague-Dawley rats were randomly divided into three experimental groups of six rats per group. The animals were fed ad libitum with the custom diet which contains 35% of energy from fat, 18% from protein, and 47% from carbohydrate[10,11]. All groups were treated via a gavage tube twice a day for 4 weeks: Group 1 (control group, n = 6) : rats were fed distilled water (8 mL/Kg BW/day), Group 2 (alcohol group, n = 6) : rats were fed 50% alcohol (8 g/Kg BW/day), Group 3 (genistein group, n = 6) : rats were fed genistein (16 mg/Kg BW/day)[12] dissolved in 50% alcohol (8 g/Kg BW/day).

Body weight of all rats was measured daily. At the end of the study, all rats were sacrificed after 12-h fasting by using intraperitoneal injection of an overdose thiopental sodium. The abdomen was opened and the whole liver was rapidly removed and washed immediately with a cold normal saline to remove as much

blood as possible. The liver was cut into several pieces. Three small pieces of the livers were collected, immediately frozen in liquid nitrogen, and stored at -80 ˚C for lipid peroxidation (MDA) and glutathione (GSH) analysis until being analyzed. The remaining of liver was excised and fixed in 10% formalin solution for histopathological analysis. Subsequently, blood sample was withdrawn by cardiac puncture. Blood samples were kept at room temperature 2 hours and then centrifuged at 1,000 g for 20 minutes. Serum samples were collected for liver enzymes testing including AST and ALT.

Histopathological examination

The liver samples were fixed in 10% formalin solution at room temperature; they were processed by routine histology procedures. Liver tissues were embedded in paraffin. Next, tissue sections at 5 µm were stained with hematoxylin and eosin (H&E), and then put on glass slides for light microscopy. An experienced pathologist blinded to the experiment evaluated all samples. All fields in each section were examined for grading of steatosis (0-3), hepatocyte ballooning (0-3) and lobular inflammation (0-3) according to the criteria described by Brunt *et al*[13].

Hepatic malondialdehyde (MDA) determination

MDA level was measured from the homogenized tissue using a commercial assay kit (Cayman Chemical, USA). The principle is measuring the rate of production of thiobarbituric acid-reactive under high temperature and acidic conditions. First of all, one gram of liver tissue was homogenized in RIPA buffer with protease inhibitor on ice by sonicate machine for fifteen seconds. Then, the liver tissue homogenates were centrifuged at 1,600 g for 10 min at 4˚C and the supernatants were collected. After following the manufacturer's protocol, the absorbance of the supernatant fraction was determined at a wavelength of 532 nm and MDA levels were calculated from a standard curve which were expressed in terms of nmol/mg protein.

Hepatic glutathione (GSH) determination

GSH level was determined from the homogenized tissue using a commercial assay kit (Cayman Chemical, USA). Liver tissues were washed with a PBS solution. Next, tissues were homogenized with cold MES buffer before centrifuge at 10,000 g for 15 minutes. Finally, the supernatants were collected and deproteinated. After following the manufacturer's protocol, the absorbance of the supernatant fraction was determined at a wavelength of 405 nm and the GSH contents were calculated in comparison with a standard GSH curve and the results were presented as nmol/mg protein.

Serum alanine transaminase (ALT) and aspartate transaminase (AST) determination

Serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were measured by colorimetric method[14] using the biochemical analyzer Reflotron®. Briefly, Serum was applied onto the test strip. Then, the strip was moved into the measuring position. Finally, the reagents contained in the test strip were contacted with the serum and started the reaction which the color reaction is measured. The results were presented liver enzymes concentration as units/ liter.

Statistical analysis

All data were presented as mean and standard deviation (SD) for continuous data and frequency for categorical data (histopathological scores). For comparison among all groups of animals, one way analysis of variance (one-way ANOVA) and Tukey's post-hoc comparisons were employed. Differences were considered statistically significant at $p<0.05$. The statistical tests were performed using the SPSS 17.0 software for windows.

RESULTS

Serum aspartate transaminase (AST) level

Serum level of AST in alcohol group was significantly higher than control group (665.17±211.35 vs. 189.15±53.69 IU/L, $p<0.01$). Nevertheless, level of AST in serum was significantly decreased by genistein administration when compared with alcohol group (283.33±137.92 vs. 665.17±211.35 IU/L, $p<0.01$) (Figure 1).

Serum alanine transaminase (ALT) level

The serum level of alanine transaminase in alcohol group was higher than control group (120.30±75.30 vs. 59.6±9.78 IU/L). However, the high elevation of serum alanine transaminase level was significantly decreased by genistein group when compared with alcohol group (32.43±12.90 vs.120.30±75.30 IU/L,

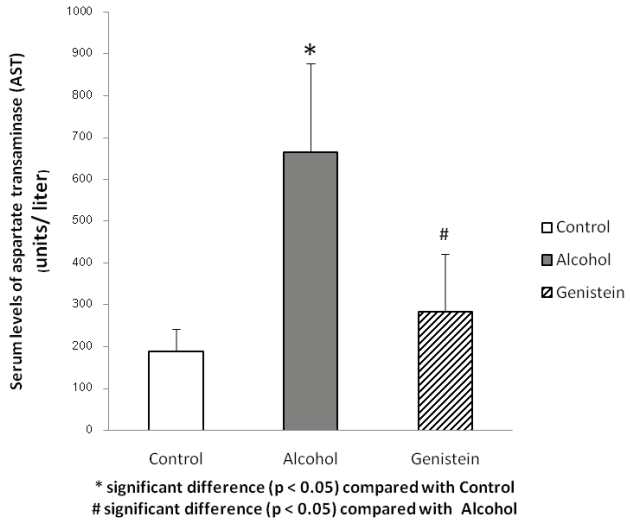


Figure 1. Serum levels of aspartate transaminase (AST) in all groups.

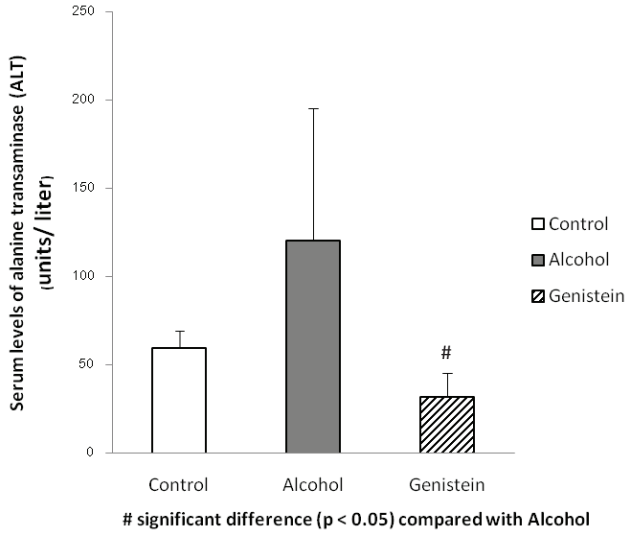


Figure 2. Serum levels of alanine transaminase (ALT) in all groups.

p<0.05) (Figure 2).

Hepatic malondialdehyde (MDA) level

The level of hepatic MDA in alcohol group was significantly higher than in control group (0.13±0.02 vs. 0.1±0.01 nmol/mg protein, p<0.05). In contrast, genistein group decreased the elevation of hepatic MDA level significantly when compared with alcohol group (0.09±0.02 vs. 0.13±0.02 nmol/mg protein, p <0.01) (Figure 3).

Hepatic glutathione (GSH) level

The level of hepatic GSH in control group was 12.66±1.08 nmol/mg protein, while alcohol group was 11.55±1.15 nmol/mg protein. Genistein group was significantly higher than in alcohol group (14.77±2.80 vs. 11.55±1.15 nmol/mg protein, p <0.05) (Figure 4).

Histopathology examination

Section of liver from alcohol group histologically exhibited liver injury features, including mild steatosis, mild lobular inflammation and mild to moderate ballooning degeneration of hepatocytes. On the other hand, genistein group improved liver histopathology when compared with alcohol group (Figure 5-8).

DISCUSSION

The spectrum of liver damage result from alcohol is not uniform. For descriptive purposes, three main histological stages of alcohol induced liver injury are

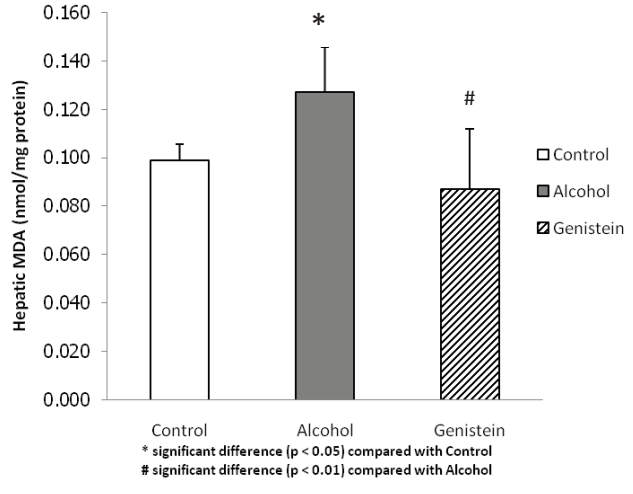


Figure 3. Hepatic MDA levels in all groups.

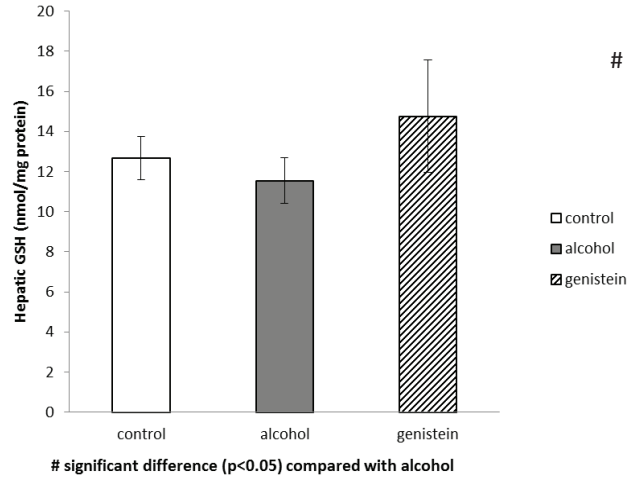


Figure 4. Hepatic GSH level in all groups.

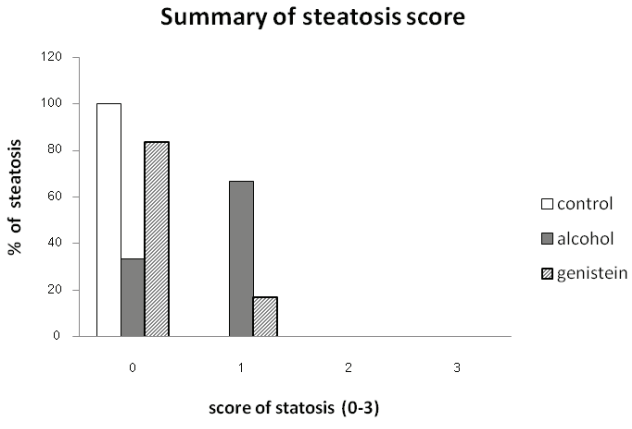


Figure 5. Summary of steatosis score.

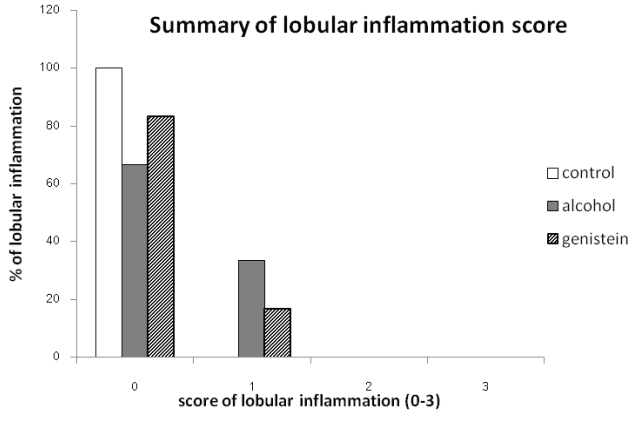


Figure 6. Summary of lobular inflammation score.

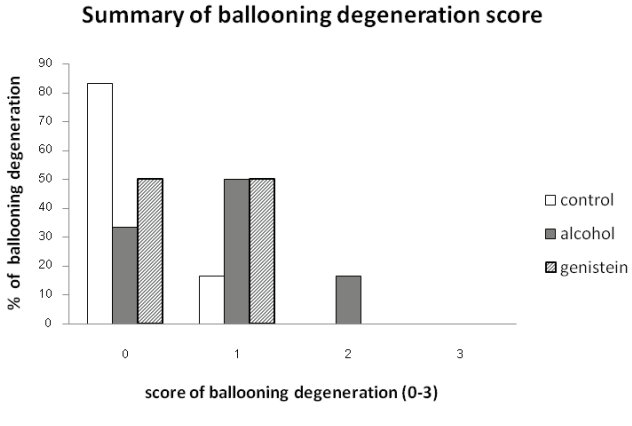
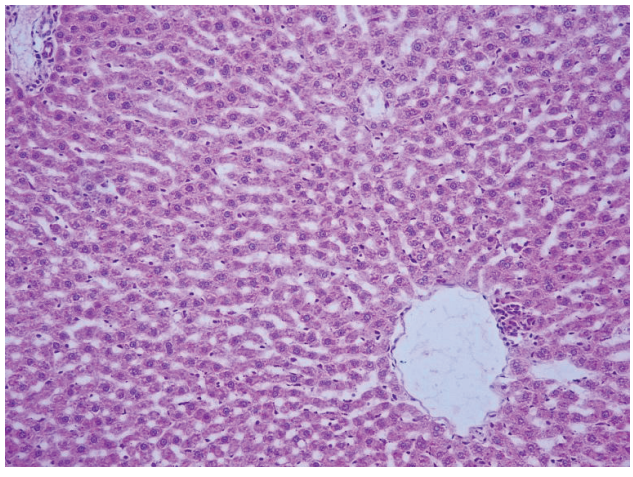
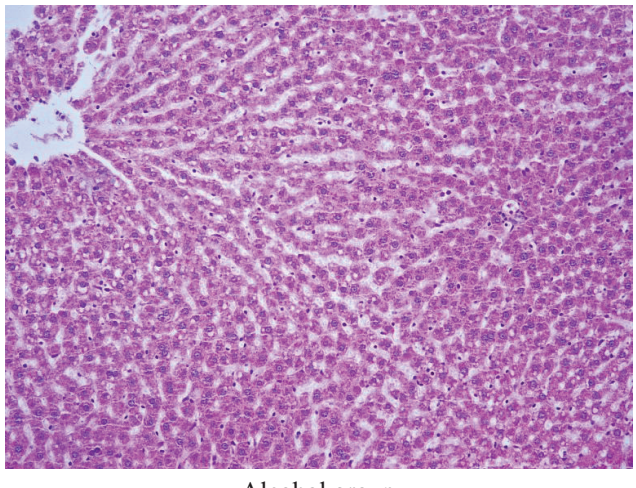


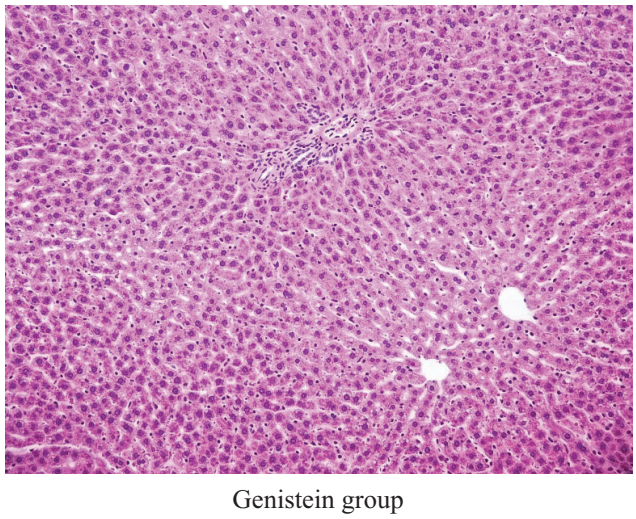
Figure 7. Summary of ballooning degeneration score.



Control group



Alcohol group



Genistein group

Figure 8. Hematoxylin-eosin stained liver sections (x400) in all groups.

steatosis (fatty liver), alcoholic hepatitis (liver injury with associated inflammation and fibrosis) and cirrhosis. In fact, these identities overlap, and it is difficult to find them separated in their pure histopathological form[15]. In the present study showed that section of liver from alcohol group histologically exhibited liver injury features, including mild steatosis, mild lobular inflammation and mild to moderate ballooning degeneration of hepatocytes; likewise, histopathologic features typically represent alcoholic liver injury. In contrast, genistein group could improve liver histopathology when compared with alcohol group; similarly, the results was showed by Huang *et al.* and Zhuo *et al.*[16, 17]. The model about ethanol-containing liquid diets is developed by Lieber and DeCarli[11] which has become important experimental equipment to reproduce in laboratory animals the physiological and pathological reformations seen in human alcoholics. Nonetheless, the diet has limited success for inducing progressive alcoholic liver injury beyond the fatty liver stage in rats. In fact, there are three important genetic characteristics that differ from human. First of all, the rats have natural aversion to ethanol. Second, the rats are higher ethanol metabolic rate than humans. Finally, the rats are greater tolerance to ethanol than man[18]. Moreover, scientific evidence showed that female rats are more weakened to early alcohol-induced liver injury than males, for instance steatosis, inflammation, and necrosis can be developed more quick and severe in females than males[19-21]. Consequently, the liver histopathologic features in this study showed early stage of liver injury when alcohol induced liver damage in male rats.

Acetaldehyde and reactive oxygen specie are the toxic products which are produced by ethanol. They have a good potential to react especially polyunsaturated fatty acids that leads to lipid peroxidation (LPO). In addition, an end-product of LPO is malonyldialdehyde (MDA) which has been widely used as a marker of oxidative stress[22]. Many studies showed that alcohol could increase the hepatic MDA level[16,17,23-25]; likewise, the result in this study showed that the level of hepatic MDA in alcohol group was significantly higher than control group. After treatment, genistein could deplete the elevation of hepatic MDA level; as a result, indicated that genistein may inhibit lipid peroxidation. Ethanol not only increases production of ROS but also depletes oxidative defense in the cell[26]. Protection of cells from ROS have several mechanisms by enzymatic and non-enzymatic[27]. Glutathione (GSH) is the most abundant tripeptidethiol in mammals as a direct against ROS and central function of detoxifying[28]. In alcoholic liver disease which produces oxidative stress leads to the deficiency of gluthathione[26]. Prior research revealed that acute alcohol intake could bring about decreasing of glutathione in the liver[24,25,29]. Besides, in the liver, a great luxuriance of antioxidant enzymes including superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase can also impair oxidative stress[30]. These data may explain the result in our study that there was a trend toward a decreased hepatic GSH level in alcohol group but not reach significance when compared with control. Moreover, in the present study, genistein administration could elevate the concentration of glutathione in the liver; in addition, it has trend to increase more than control group. In fact, Nrf2 regulates ARE-dependent gene regulation which can bind together in the nucleus for activation antioxidant enzyme gene transcription such as glutathione regulatory enzymes[31-34]. Thus, the result in this study might be due to genistein could increase gluthathione level by activation Nrf2 expression which accorded the study by Ma *et al.*[35]. Finally, the result of decreasing hepatic MDA level described above, we indicated that genistein can protect oxidative stress when ethanol intake by restoration level of hepatic glutathione.

Analysis of serum enzymes is one in every of the foremost sensitive tests used within the diagnosis of liver injury. Alanine aminotransferase (ALT) is located in cytoplasm while aspartate aminotransferase (AST) is located in cytoplasm and mitochondrial; moreover, both enzymes will be discharged into blood circulation once hepatocyte structural integrity harm[36,37]. In the present study, levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were increased in alcohol group when compared with control group; nonetheless, the difference level of ALT in alcohol group did not reach a statistical significance. Likewise, previous report has shown by Dahiru and Obidoa which serum level of ALT was not increased significance when treated rat with ethanol via oral for 6 weeks[36]. Alcohol liver injury could be related to decrease ALT, it is due to pyridoxine deficiency[38]. In contrast, levels of serum ALT and AST were decreased when we treated with genistein. Similarly, the recent study, Zhuo L *et al.* found that combination therapeutics with taurine, epigallocatechingallate and genistein

on liver fibrosis when treated alcohol in rats could decrease levels of serum AST and ALT[17]; likewise, the study by Huang Q *et al.* revealed that genistein isolated from hydrocotylesibthorpioides on liver injury and fibrosis induced by chronic alcohol in rats could decrease levels of serum ALT and AST[16]. The results of liver histopathology described above, we hypothesize that alcohol-induced liver injury in rats when treated with genistein can preserve the structural completeness of the liver.

CONCLUSION

The present study found that genistein could improve liver histopathology and liver enzymes by decreasing oxidative stress and increasing glutathione level in rats with alcohol-induced liver injury.

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