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Histopathological and Immunohistochemical Features of the Liver of Mice by Giving Temulawak Rhizome Extract (*Curcuma xanthorrhiza* Roxb.)

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Abstract

This study aims to determine the levels of ALT, AST, histopathological, and immunohistochemical in mice livers after administration of Temulawak rhizome extract. In the liver protection power, the test preparations were administered for 7 consecutive days, on the 8th day the animals were induced with a CCl_4 dose of 0.014 mg/20 g body weight, and on the 9th day blood and liver samples were taken to measure ALT and AST levels and to observe histopathological and immunohistochemical. In the recovery power, on the first day, animals were induced with CCl_4 at a dose of 0.014 mg/20 g body weight, on the 2nd to 8th day, each group was given a test preparation, and on the 9th day, blood and liver samples were taken to measure ALT and AST levels and to observe histopathology and immunohistochemical. The results of the protective power test showed that the average ALT and AST levels in the aquadest, silymarin, and Temulawak rhizome extract groups were 36.82±1.04; 19.02±0.57; and 19.75±0.85 ($p<0.05$) and 72.58±3.23; 37.46±0.83; and 37.95±1.32 ($p<0.05$), respectively. The liver recovery power test results showed the average ALT and AST levels in the aquadest, silymarin, and Temulawak rhizome extract groups were 33.02±1.41; 21.61±0.51; and 21.93±0.57 ($p<0.05$) and 69.75±1.05; 37.38±1.27; and 38.11±0.74 ($p<0.05$), respectively. The antioxidant level of Temulawak rhizome extract in the protective power test gave a strong reaction, while in the recovery power test, it gave a moderate reaction. Immunohistochemical of Temulawak rhizome extract for protection and recovery test showed a weak brown color intensity.

Major Findings: The most significant findings of this research is giving therapeutic doses of Temulawak rhizome extract has protection and restoration of liver function which is characterized by a decrease in ALT and AST activity. In the protective potency test, therapeutic doses of Temulawak rhizome extract had a strong antioxidant level.

Keywords: Hepatoprotector, Histopathology, Immunohistochemical, Liver, Temulawak

1. Introduction

Almost the majority of Indonesian local people still use medicinal plants as herbal ingredients for various diseases[1-4]. The use of plants as medicine has been passed down from generation to generation. These people usually use plants grown around the house, or wild in the forest, or cultivated or on special land for medicinal plants[4]. There are many benefits of plants in medicine, including as an appetite enhancer[3],

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increasing body fitness[5], antidiabetic mellitus[6,7], and hepatoprotector[8-10].

Hepatoprotection is a compound that can protect the liver from damage caused by toxic substances such as drugs, hepatitis viruses, and unhealthy lifestyles such as smoking and drinking alcohol which poison the liver[11]. Many hepatoprotection compounds are obtained from medicinal plants that contain natural antioxidant compounds such as flavonoids, terpenoids, and steroids or triterpenoids[12]. The liver is the largest and most complex internal organ in the body. The liver has an important role in maintaining the internal environment of the body with various functions such as protein, fat, and carbohydrate metabolism and in the synthesis of plasma proteins such as albumin, fibrinogen, and in the production of various enzymes, the formation and excretion of bile[13,14]. In connection with this function, the liver is often exposed to compounds that enter the body, even these compounds can cause liver damage[15,16]. Some researchers report that liver damage can be caused by consuming drugs such as paracetamol[17,18], viral hepatitis[19], and an unhealthy lifestyle due to consuming alcoholic beverages[20]. Liver damage is characterized by increased serum levels of the enzymes Aspartate Transaminase (AST) and Alanine Transaminase (ALT)[18,21-26], histopathological features characterized by steatosis in liver cells, necrosis, fatty degeneration, and hydropic degeneration[21,23-25,27], as well as immunohistochemical features[27-29]. Observation of the immunohistochemical features of the liver using Cu, Zn-SOD staining to detect Cu, Zn-SOD producing cells which can indicate the number of producing cells and the antioxidant content Cu, Zn-SOD, which is characterized by the intensity of the brown color indicating the content of Cu, Zn-SOD, the darker and more even the brown color means that it contains more Cu, Zn-SOD, and gives a positive reaction at various levels of content towards Cu, Zn-SOD (dark brown or strong positive or +++, medium brown or moderate positive or ++, and bluish light brown or weak positive or +/-, and blue or negative or -)[28,29].

Temulawak (*Curcuma xanthorrhiza* Roxb.) is a plant from the *Zingiberaceae* family. Temulawak flower is reported to contain several secondary metabolites such as xanthorrhizol (16.13%), α-curcumene (15.12%), β-elemene (4.60%), trans-caryophyllene (3.48%), β-farnesene (0.29%), camphor (0.21%), and isoborneol (0.04%) so that Temulawak flowers can be used for skin care[30]. Temulawak leaves were reported to contain several secondary metabolites such as germacrone (18.76%), xanthorrhizol (12.22%), germacrene B (7.19%), eucalyptol (6.89%), γ-eudesmol acetate (6.33%), ar-curcumene (6.06%), β-himachalene (5.75%), and curzerene (5.24%)[31] so that Temulawak leaves can be used as gastroprotectors[32]. Temulawak rhizome is reported to contain several secondary metabolites such as xanthorrhizol (64.38%), camphene (8.27%), curcumin (5.85%), α-pinene (1.93%), α-thujene (0.16%), β-pinene (0.14%), myrcene (0.37%), linalool (0.27%), and zingiberene (0.10%)[33]. Temulawak rhizome is reported to have properties as an antioxidant[34,35], anti-diabetic[36,37], anti-inflammatory[36,38], anticancer[39,40], antibacterial[41,42], skin care[43], and hepatoprotector[18,21,44].

So far, testing the hepatoprotective power of Temulawak rhizome according to Lin *et al*., using a dose of 100 mg/kg can reduce AST and ALT levels in rats induced by acetaminophen and CCl_4 and suppress centrilobular necrosis around the central vein and hepatic binuclear cells are found in the middle zone, which shows regeneration of hepatocytes in histopathology of liver organs[44]. Devaraj *et al*. reported that treatment with the hexane fraction of standard Temulawak rhizome at 500 mg/kg for 7 consecutive days reduced ALT and AST levels by 40-80% respectively[21]. Based on the results of the histopathological examination of the liver, the histological architecture of the liver sections of the rats in the treated group with the standard Temulawak rhizome hexane fraction at 500 mg/kg showed an almost normal lobular pattern with a low level of necrosis, clear cells, and visible reticular fibers around the central vein were almost comparable with the control and silymarin groups. According to Masfufatun *et al.,* administration of Temulawak ethanol extract at a dose of 1080 mg/200 g body weight reduced the activity of ALT and AST enzymes in the blood serum of paracetamol-induced rats[18]. However, an effective dose related to hepatoprotector testing has not been found. This study reports the levels of ALT, AST, histopathology, and immunohistochemical at therapeutic doses. A therapeutic dose or therapeutic dose is the dose given under normal circumstances and can cure. The therapeutic dose is relatively far from the lethal dose. In consuming and using it, it is better if the dose of the test substance is not close to the lethal dose because it can cause poisoning to death (General Provisions of the Indonesian Pharmacopoeia Edition III, 1979).

Considering the potential of Temulawak rhizome and its wide use as medicine, the hepatoprotection activity of

Temulawak rhizome was tested in terms of ALT, AST levels, histopathology, and immunohistochemical. It is hoped that the results of this study can provide information and enrich knowledge about the hepatoprotection activity of Temulawak rhizome itself.

2. Materials and Methods

2.1 Extraction Process

Extraction of Temulawak rhizomes refers to the method of Harborne. Temulawak rhizomes are cleaned using water, then dried and chopped. Temulawak was weighed and the wet weight was 1.33 kg, then it was dried in the sun and the dry weight was 395 g. As much as 395 g of dried Temulawak rhizomes were macerated using 96% ethanol. Maceration was carried out 3 times, each for 24 hours. Furthermore, concentration was carried out using an evaporator and 54 g of extract was obtained[45].

2.2 Preparation of Test Animals

The test animals used were male white mice (*Mus musculus*), weighing 22-26 g, in healthy condition, with an age range of ± 8 weeks. Before the experiment, all test animals were acclimatized for 7 days by providing standard food and drink and libitum. The testing procedure has received a statement letter from the Health Research Ethics Commission, Faculty of Health Sciences, Yogyakarta Respati University No. 019.3/FIKES/PL/I/2021.

2.3 Hepatoprotector Activity Test

The research was divided into 2 parts, namely the liver's protective power and the liver's recovery power. In the study of liver protection, 18 mice as test animals were divided into 3 groups, each group consisting of 6 mice. The test material given to each group; Group 1 was given aquadest at a dose of 0.056 ml/20 g body weight, Group 2 was given silymarin at a dose of 0.7 mg/20 g body weight, and Group 3 was given Temulawak rhizome ethanol extract at a dose therapeutic. Provision of test material was carried out for 7 consecutive days. On the 8th day, all mice in each group were induced with CCl_4 at a dose of 0.014 mg/20 g body weight. On the 9th day, all mice in each group were sacrificed and their livers were removed for histopathological and immunohistochemical examination. In the study of liver recovery power, 18 mice as test animals were divided into 3 groups, each group consisting of 6 mice. The test material given to each group; Group 1 was given aquadest at a dose of 0.056 ml/20 g body weight, Group 2 was given silymarin at a dose of 0.7 mg/20 g body weight, and Group 3 was given Temulawak rhizome ethanol extract at a dose therapeutic. Before administration of the test substance, on day 1, all mice in each group were induced by CCl_4 at a dose of 0.014 mg/20 g body weight. On the 2nd to 8th day in a row, the test material was administered to each group. On the 9th day, the blood and liver samples were taken. Blood samples were taken from the orbital sinuses of mice. The blood obtained was then centrifuged for 15 minutes at 4000 rpm. Next, the serum was separated and put into the eppendorf tube. After that, the levels of the enzymes Alanine Transaminase (ALT), and Aspartate Transaminase (AST) were measured. ALT and AST levels were measured using a kit (DiaSys) with the UV-Test method. Data on ALT and AST levels were statistically analyzed using SPSS Statistics 17.0, using the Tukey test. The difference was considered significant at $p<0.05$.

2.4 Observation of Histopathological Features

Animals were sacrificed by cervical dislocation, then necropsy was performed for macroscopic organ evaluation, followed by histopathological examination. The livers taken were routinely processed, and then stained with Hematoxylin-Eosin (HE)[46]. Histopathological staining results were observed under a light microscope. Changes that appear are carried out by giving a score, namely: A score of 0, no specific changes are found; score 1, liver cells experience hydropic degeneration and a mild degree of fatty degeneration, evenly distributed; score 2, liver cells have a moderate degree of fatty degeneration and steatosis, are multifocal; and score 3, liver cells have fatty degeneration, steatosis, and severe dystrophy, are multifocal.

2.5 Immunohistochemical Observation

Animals were sacrificed by cervical dislocation, then a necropsy was performed for macroscopic organ evaluation, followed by an immunohistochemical examination. Liver organs taken were routinely processed, and then Cu, Zn-SOD detection was carried out by immunohistochemical. This study used a dako polymer peroxidase secondary antibody (Dako K1491). After deparaffinization and rehydration, the tissue

sections were treated with 3% H_2O_2 for 10 minutes to remove endogenous peroxidase activity. Then the tissue sections were incubated in 10% normal serum for 30 minutes at 37^{0}C. The tissue sections were then washed with phosphate buffer before incubating in the primary antibody, namely Cu, Zn-SOD monoclonal antibody (Sigma S2147) at 4^{0}C. The tissue sections were incubated again and washed with phosphate buffer. Then the tissue sections were incubated again and washed with phosphate buffer. The product of the antigen-antibody reaction in tissue sections was visualized with Diamino Benzidine (DAB: 1 mL high concentration of DAB substrate mixed with 50 µL/1 drop of DAB Chromogen) for 25 minutes at room temperature. Then wash with running water and counterstain with hematoxylin. Wash with running water, then soak with bluing reagent and wash with running water. Furthermore, dehydration was carried out with graded alcohol and clearing with xilol, and closing the tissue pieces with a cover slip using adhesive. As a control, tissue sections were incubated using phosphate buffer instead of Cu, Zn-SOD monoclonal antibodies. Observation of Cu, Zn-SOD producing cells was carried out on positive reaction products in the cytoplasm of liver cells by comparing the intensity of the brown color formed and its distribution to all parts of each preparation that were observed. The intensity of the brown color indicates the content of Cu, Zn-SOD, the darker and more even the brown color means that it contains more Cu, Zn-SOD[27].

3. Results

Table 1 shows that in the protective power test the average levels of ALT and AST in the aquadest group, the silymarin group, and the Temulawak rhizome extract group showed significant differences ($p<0.05$), on the other hand, the silymarin and extract groups were Temulawak rhizome was not significantly different ($p>0.05$). This shows that administration of silymarin at a dose of 0.7 mg/20 g body weight and therapeutic doses of Temulawak rhizome extract has a protective effect by lowering ALT and AST levels compared to administration of aquadest at a dose of 0.056 ml/20 g body weight. Table 1 shows that in the recovery power test the average levels of ALT and AST in the aquadest group, the silymarin group, and the Temulawak rhizome extract group showed significant differences ($p<0.05$), on the other hand, the silymarin and extract groups were Temulawak rhizome was not significantly different ($p>0.05$). This shows that administration of silymarin at a dose of 0.7 mg/20 g body weight and therapeutic doses of Temulawak rhizome extract have healing power by lowering ALT and AST levels compared to administration of aquadest at a dose of 0.056 ml/20 g body weight.

Table 2 showed that in the protective power test the observed results of the antioxidant content of Cu, Zn-SOD in 5 visual fields in the group given silymarin at a dose of 0.7 mg/20 g body weight and the group given Temulawak

Table 2. The average levels of Cu, Zn-SOD antioxidants in each field of view on the test of liver function protection and recovery

Treatment Group	Cu, Zn-SOD Antioxidant Content Levels at 5 Fields of View	
	Protection Power Test	Recovery Power Test
Aquadest	++	++
Silymarin	+++	+++
Temulawak Rhizome Extract	+++	++

Information:
(-) = Negative
(+) = Weak
(++) = Moderate
(+++) = Strong

Table 1. The results of the test for protection and recovery of liver function by measuring the average levels of ALT and AST in mice given the test materials, namely aquadest, silymarin, and Temulawak rhizome extract

Treatment Group	Protection Power Test		Recovery Power Test	
	ALT	AST	ALT	AST
Aquadest	36.82^b±1.04	72.58^b±3.23	33.02^b±1.41	69.75^b±1.05
Silymarin	19.02^a±0.57	37.46^a±0.83	21.61^a±0.51	37.38^a±1.27
Temulawak Rhizome Extract	19.75^a±0.85	37.95^a±1.32	21.93^a±0.57	38.11^a±0.74

Data is displayed in the form of Mean±Standard Deviation (SD)
Groups given the same letter were not significantly different ($p>0.05$)

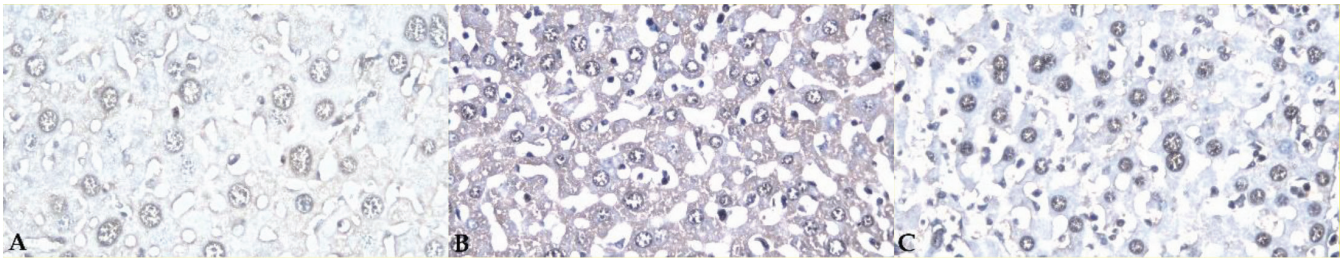


Figure 1. Immunohistochemical features in the liver function protection test: **(A)** The group given aquadest at a dose of 0.056 ml/20 g body weight **(B)** The group given silymarin at a dose of 0.7 mg/20 g body weight **(C)** The group given the therapeutic dose of Temulawak rhizome extract (magnification 400x).

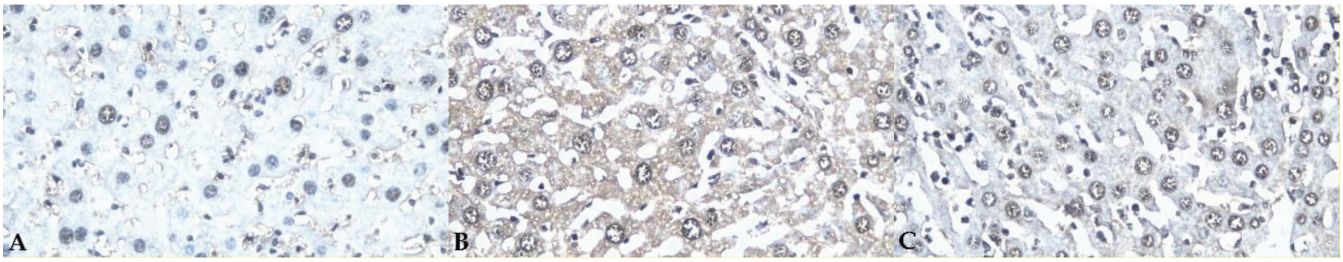


Figure 2. Immunohistochemical features in the liver function recovery test **(A)** The group given aquadest at a dose of 0.056 ml/20 g body weight **(B)** The group given silymarin at a dose of 0.7 mg/20 g body weight **(C)** The group given the therapeutic dose of Temulawak rhizome extract (magnification 400x).

rhizome extract therapeutic doses were at strong levels, while the antioxidant content Cu, Zn-SOD in each visual field in the 0.056 ml/20 g body weight aquadest group was at a moderate level. In the recovery power test, the results of observations on the antioxidant content of Cu, Zn-SOD in 5 visual fields in the group given silymarin at a dose of 0.7 mg/20 g body weight was at a strong level, while the antioxidant content of Cu, Zn-SOD in each group's field of view the therapeutic dose of Temulawak rhizome extract and the group that was given aquadest at a dose of 0.056 ml/20 g body weight were at moderate levels.

Figure 1 showed that the liver function protection test for the observation of the antioxidant content of Cu,Zn-SOD, showed a decrease in the antioxidant content in the liver tissue of mice in the group given aquadest and Temulawak rhizome extract. The decrease in antioxidant content in the aquadest group was evident from the absence of the intensity of the brown color formed in both the nucleus and cytoplasm of the liver cells. In contrast, the decrease in antioxidant content in the Temulawak rhizome extract group was evident from the weak brown color intensity in both the nucleus and cytoplasm of the liver cells. The antioxidant content of the silymarin group was seen to increase which was marked by a strong brown color intensity in both the nucleus and cytoplasm of liver cells. Figure 2 showed that the liver function recovery test for the observation of the antioxidant content of Cu, Zn-SOD, showed a decrease in the antioxidant content in the liver tissue of mice in the group given aquadest and Temulawak rhizome extract. The decrease in the antioxidant content in the aquadest group was seen from the absence of the intensity of the brown color formed in both the nucleus and the cytoplasm of the liver cells, while the decrease in the antioxidant content in the group receiving the Temulawak rhizome extract was evident from the weak intensity of the brown color in both the nucleus and the cytoplasm of the cells. On the other hand, the antioxidant content of the silymarin group increased as indicated by the strong brown color intensity in both the nucleus and cytoplasm of the liver cells.

4. Discussion

The liver is the most complex organ in the body and has an important role in the metabolism of proteins, fats, and carbohydrates, as well as in the synthesis of plasma proteins such as albumin, fibrinogen, and in the

production of various enzymes, formation and excretion of bile[13,14]. However, the liver has the potential to be damaged and can interfere with the body's metabolism, causing liver disease. Damage to the liver can be caused by the activity of chemical compounds in the form of drugs that enter the body in large quantities and are exposed for a long time, this can result in impaired liver detoxification function[47]. Chemical compounds that are efficacious in protecting liver cells as well as overcome damage to liver tissue from the effects of toxic substances are called hepatoprotections. Hepatoprotection is a compound that can protect the liver from damage caused by toxic substances such as drugs, hepatitis viruses, and unhealthy lifestyles such as smoking and drinking alcohol so that it poisons the liver[11].

Many hepatoprotection compounds are obtained from medicinal plants that contain natural antioxidant compounds such as flavonoids, terpenoids, and steroids or triterpenoids[12]. Liver damage is characterized by increased serum levels of the enzymes Aspartate Transaminase (AST) and Alanine Transaminase (ALT)[18,21-26]. Alanine transaminase is an enzyme present in the liver cytosol and involved in glucogenesis. This enzyme plays a role in catalyzing the transfer of amino groups from alanine to alpha-ketoglutaric acid to form glutamic acid and pyruvic acid. The increased activity of ALT enzymes in the blood is mainly caused by damage to liver cells and skeletal muscle cells. Liver damage begins with changes in membrane permeability followed by cell death[48]. Aspartate transaminase is an enzyme found in liver parenchyma cells. This enzyme can be found in the heart, skeletal muscle, and kidneys. When the tissue is acutely damaged, its serum level increases. Elevated levels of AST occur due to the release of enzymes intracellularly into the blood caused by necrosis of liver cells or acute liver damage[49]. Under normal conditions, in serum, ALT enzyme levels are lower than AST[50].

Based on the results of this study, administration of CCl_4 at a dose of 0.014 mg/20 g body weight can cause damage to liver function as indicated by increased levels of ALT and AST enzymes in the group given aquadest at a dose of 0.056 ml/20 g body weight. This is because CCl_4 is a xenobiotic that is commonly used to induce lipid peroxidation and poisoning. In the endoplasmic reticulum of the liver, CCl_4 is metabolized by cytochrome P450 2E1 (CYP2E1) to become trichloromethyl free radical (CCl_3). Trichloromethyl with oxygen will form trichloromethylperoxi radicals which can attack endoplasmic reticulum membrane lipids at a rate that exceeds trichloromethyl free radicals. Furthermore, trichloromethylperoxi causes lipid peroxidation thereby disrupting Ca^{2+} homeostasis, and ultimately causing cell death[51]. In addition, aquadest cannot provide protection or recovery power to the liver because aquadest is inert (does not react chemically or biologically) so does not affect mice.

The results showed that administration of silymarin at a dose of 0.7 mg/20 g body weight had protection and restoration of liver function by reducing ALT and AST levels. This is because silymarin is included in the class of flavonoids (silybin, isosilybin, silychristin, silydianin, and taksifolin)[52]. Silymarin is contained in the dried fruit of the plant *Silybum marianum*. *S. marianum* is a plant from the *Asteraceae* family. The *S. marianum* plant has been used for 2000 years for the treatment of liver disorders. In addition, it is also used in European countries to treat biliary disorders and alcoholic liver disease. The use of this plant is increasing in other countries including the treatment of cirrhosis, hepatitis C, and the prevention of liver toxicity[53]. Silymarin is reported to have antioxidant potency and function as a hepatoprotector[23,25]. Silymarin also acts as anticancer[54,55], anti-inflamation[53,56], antidiabetic[57], and immunomodulators[53].

The results showed that administration of Temulawak rhizome extract had protection and restoration of liver function by reducing ALT and AST levels, not significantly different from silymarin administration at a dose of 0.7 mg/20 g body weight ($p>0.05$). This is because the Temulawak rhizome has hepatoprotective properties[18,21,44]. In addition, Temulawak rhizome is also reported to contain phytochemical compounds in the form of alkaloids, tannins, saponins, flavonoids, and steroids or triterpenoids, whereby flavonoids and steroids or triterpenoids are antioxidant compounds that are hepatoprotective agents[12]. Alkaloids have antioxidant properties by inhibiting oxidation reactions so that the formation of free radical chains is inhibited using proton donors to stabilize free radicals[58]. Saponins are efficacious as antidiabetics because they are inhibitors of the α-glucosidase enzyme and tannins have an important role in reducing blood glucose levels[59].

In the immunohistochemical observations, calculations were made of liver cell nuclei which gave positive reactions at various levels of Cu, Zn-SOD content, and those which gave negative reactions to these antioxidants. The results of counting the liver cell nuclei in

the liver function protection test showed that the aquadest group gave a moderate reaction, while the silymarin and Temulawak rhizome extract group gave a strong reaction. This shows that the antioxidant content in the livers of mice that were given silymarin and Temulawak rhizome extract after administration of CCl_4 was more than those that were only given aquadest. Meanwhile, the results of counting liver cell nuclei in the liver function recovery test showed that the group given aquadest and Temulawak rhizome extract gave a moderate reaction, while the group given silymarin gave a strong reaction. This shows that the antioxidant content in the livers of mice that were given silymarin before administration of CCl_4 was more than those that were only given aquadest and Temulawak rhizome extract.

By immunohistochemical staining, it was seen that Cu, Zn-SOD producing cells in liver tissue gave a positive reaction to these antioxidants. The product of a positive reaction to Cu, Zn-SOD which gave a brown color was seen in both the cytoplasm and the nucleus of the liver cells[25]. In the liver function protection test for the observation of the antioxidant content of Cu, Zn-SOD, it showed a decrease in the antioxidant content in the liver tissue of mice in the aquadest and Temulawak rhizome extract group. The decrease in the antioxidant content in the aquadest group was evident from the absence of the intensity of the brown color formed in both the nucleus and the cytoplasm of the liver cells, while the decrease in the antioxidant content in the group given the Temulawak rhizome extract was evident from the weak intensity of the brown color in both the nucleus and the cytoplasm of the liver cells. On the contrary, the antioxidant content of the silymarin group increased which was indicated by a strong brown color intensity in both the nucleus and cytoplasm of liver cells. In the liver function recovery test for the observation of the antioxidant content of Cu, Zn-SOD, it was shown that there was a decrease in the antioxidant content in the liver tissue of mice in the group given with aquadest and Temulawak rhizome extract. The decrease in the antioxidant content in the aquadest group was evident from the absence of the intensity of the brown color formed in both the nucleus and the cytoplasm of the liver cells, while the decrease in the antioxidant content in the group given the Temulawak rhizome extract was evident from the weak intensity of the brown color in both the nucleus and the cytoplasm of the liver cells. In contrast, the antioxidant content of the silymarin group increased as indicated by the strong brown color intensity in both the nucleus and cytoplasm of the liver cells. This shows that administration of silymarin at a dose of 0.7 mg/20 g body weight has better antioxidant potency than therapeutic doses of Temulawak rhizome extract.

5. Conclusions

Giving therapeutic doses of Temulawak rhizome extract has protection and restoration of liver function which is characterized by a decrease in ALT and AST activity, also histopathologycal and immunohistochemical features. In the protective potency test, therapeutic doses of Temulawak rhizome extract had a strong activity.

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