HISTOLOGY OF LIVER AND KIDNEY OF WHITE RAT *(RATTUS NORVEGICUS)* INDUCED BY CARBON TETRACHLORIDE AFTER ADMINISTRATION OF COWPEA TEMPEH EXTRACT *(VIGNA UNGUICULATA)*

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Abstract. Free radical formation, triggered by continuous carbon tetrachloride (CCl_4), could cause oxidative stress that have the potential to damage the liver and kidneys. CCl_4 is still widely used nowadays, such as in the fire extinguishers, pesticides, and refrigeration industries. Cowpea (*Vigna unguiculata*) tempeh, a fermented food used as an alternative functional food, contains antioxidant compounds that can prevent the formation of free radicals. This study aimed to determine the effect of cowpea tempeh extract on the liver and kidney histology of white rats (*Rattus norvegicus*) induced by CCl_4. This study used a completely randomized design with 28 male rats grouped into 4 treatment groups, namely negative control (K-), positive control (K+) induced by CCl_4 at 1 ml/200g body weight intraperitoneally, and two treatments induced by CCl_4 at 1 ml/200g body weight and given a dose of cowpea tempeh extract at a dose of 6% tempeh ethanol extract (P1), and 9% tempeh ethanol extract (P2). Parameters observed in liver histology were fatty degeneration, hydropic degeneration, necrosis, congestion, and inflammatory cell infiltration. Parameters observed in renal histology were fatty degeneration, necrosis, inflammatory cell infiltration, and glomerular swelling. The results showed that there was a significantly different decrease in liver and kidney damage ($p<0.05$) in P1 and P2 compared to the K+ group. It can be concluded that cowpea tempeh extract was able to repair liver and kidney damage due to CCl_4-induced oxidative stress.

Keywords: carbon tetrachloride, cowpea tempeh, histology, liver, kidney

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INTRODUCTION

Liver is the largest gland in the body and is very important in regulating body homeostasis including metabolism, biotransformation, synthesis, storage, and immunology (Rafsanjani *et al*, 2018). The liver is susceptible to damages caused by several factors, one of which is oxidative stress. Oxidative stress is defined as an imbalance condition between free radicals and antioxidants in terms of maintaining conditions against tissue damage that occurs (Arief and Widodo, 2016).

Oxidative stress, not only causes damage to the liver but also to the kidneys (Hamed *et al*, 2012). Kidneys are organs that function to filter and remove waste products from the body's metabolism, making them susceptible to damage due to exposure to toxic substances. One of the toxic substances that can cause oxidative stress to cause damage is carbon tetrachloride (CCl_4) (Sediarso *et al*, 2018).

Carbon tetrachloride is a halogen aliphatic hydrocarbon compound which is widely used as a pesticide, fire extinguisher, refrigerant, and cleaning clothes (Lieberman and Peet, 2022). According to Timbrell (2008), CCl_4 is one of the compounds that often induces liver and kidney damage. Metabolism of CCl_4 with the help of cytochrome P450 enzyme catalysts in the liver produces trichloromethyl free radicals (CCl_3).

Increased production of free radicals can cause damage to various target organs such as steatosis, centrilobular necrosis and cirrhosis in the liver and acute tubular necrosis (ATN) in the kidneys (Hendra *et al*, 2014). Free radicals can be neutralized by using antioxidants. In recent years, non-soybean-based tempeh has been developed. Cowpea (*Vigna unguiculata*

subsp unguiculata) can be chosen as an option to replace soybeans to be made as tempeh considering that cowpeas have a variety of benefits and good nutritional value for the body. The process of making cowpea tempeh does not differ from making tempeh in general, only by replacing soybeans with cowpeas.

Cowpea is a legume that is widely known and used in Indonesia. Apart from being called stump, these beans are also known as *tolo beans, dadap beans,* and others. Cowpea seed extract can inhibit the growth of pathogenic bacteria and fungi (Jayathilake *et al*, 2018). Other studies have shown that cowpea extract can act as antidiabetic, anti-inflammatory, anticancer, antihypertensive, and antihyperlipidemic (Budianti, 2018). Cowpeas contain 22-30% protein, 33-59.9% carbohydrates, and 2.10-2.98% crude fiber (Gonçalves *et al*, 2016). Other studies have shown that 100 g of mature cowpea seeds contain 10 g of water; 22 g of protein; 1.4 g of fat; 51 g of carbohydrates; 3.7 g ofvitamins; 3.7 g of carbon; 104 mg of calcium and other nutrients. The energy produced is around 1420 kJ/100 g or equivalent to 339 kcal/100g (Fadillah and Purnamawati, 2020).

Based on the description above and in terms of the many benefits of cowpea tempeh, it is necessary to conduct a study to determine the effect of cowpea tempeh extract on the histological image of the liver and kidneys of white rats induced by carbon tetrachloride.

MATERIALS AND METHODS

Study design

This study was an experimental study using a completely randomized design consisting of four treatments, namely negative control (K-), positive control (K+); induced by carbon tetrachloride (CCl_4) 1 ml/200g body weight intraperitoneally, treatment group 1 (P1), and treatment group 2 (P2). P1 and P2 groups was given the cowpea tempeh extract for 7 days orally.

On Day 7, rats were induced with CCl_4 of 1ml/200g body weight intraperitoneally. Then tempeh extract was given to the rats again on the 8^{th} day to the 15^{th} day. P1 group was given 6% tempeh extract and P2 group 9% tempeh extract which in accordance with previous study (Sumbayak and Vebriyani, 2019).

Experimental animal preparation

Twenty-eight white rats (Rattus norvegicus strain Wistar) weighed 200-300 grams were the subjects of this study. The experimental animals collected were having acclimation period for one week by given regular feed and distilled water. Experimental animals that had been adapted for one week were randomly grouped into 4 groups (7 rats in each group). This study received a Letter of Ethics No. 105-KEP-UB-2022 from Universitas Brawijaya on 13 August 2022.

Tools and materials

Food dehydrator (Lequip LD-401SP – Getra, Beijing, PR China), digital scale (BC-500, ACIS, Jakarta, Indonesia), rotary evaporator (RV 3 V, IKA, Stauven, Germany), Image Raster application and OptiLab viewer application (V 2, Miconos, Jogjakarta, Indonesia), CCl_4 (Merck, Taufkirchen, Germany), coconut oil (Coco Milagro, Jakarta, Indonesia), ketamine hydrochloric acid (HCl) (Merck, Taufkirchen, Germany), and NBF (Neutral Buffered Formalin) solution (Sigma Aldrich, St Louis, MO).

Cowpea tempeh production

The process of making cowpea and flour tempeh was carried out in the Food Administration Laboratory, Department of Nutrition, Faculty of Health, Universitas Brawijaya for two days. Cowpeas were cleaned, boiled

in water for 15 minutes then cooled. After cooling, cowpeas were then given tempeh yeast, covered with banana leaves and then left for 35 and 45 hours at room temperature for the fermentation process.

Cowpea tempeh flour production

The finished cowpea tempeh was chopped into small pieces to speed up the drying process. The cowpea tempeh pieces were then put in a food dehydrator (Lequip LD-401SP, Getra, Beijing, PR China) at 60°C for 1.5 hours to dry. After the tempeh was dry, it was refined into flour using a grinder and sifted using an 80-mesh sieve to obtain cowpea tempeh flour. The finished flour then put into plastic and vacuumed and then stored for laboratory testing.

Preparation of cowpea tempeh ethanol extract

The cowpea tempeh flour was weighed as much as 150 g with a digital scale (ACIS, Jakarta, Indonesia). Soursop leaf powder was then put into a container and a maceration process was carried out by soaking the powder in 1,500 ml of ethanol with a ratio of 1:10 (w/v). The container was then tightly closed and stored at room temperature for 5 days. The extract was then filtered with gauze and filter paper and put into a bottle. The next step was the evaporation process using a rotary evaporator (IKA, Stauven, Germany) at a temperature of 40°C to obtain a crude extract (Sudirga, 2015).

Preparation of CCl_4 Solution

CCl_4 solution was prepared by dissolving 0.007 ml of CCl_4 (Merck, Taufkirchen, Germany) in 0.1 ml of coconut oil (Coco Milagro, Jakarta, Indonesia). The solution was then stirred until homogeneous and ready to use (Sumbayak and Vebriyani, 2019).

In vivo treatment

After treatment, white rats were anesthetized on Day 25 with ketamine (Merck, Taufkirchen, Germany) at a dose of 100 mg/kg body weight (Tsukamoto *et al*, 2015). The anesthetized white rat was then dislocated in the neck and dissected to remove the liver and kidneys. The organs were then washed with 0.9% NaCl solution and dried with filter paper and weighed. After being weighed, the organs were then immersed in 10% NBF (Neutral Buffered Formalin) solution (Sigma Aldrich, St Louis, MO) for preservation prior to preparation of histological incisions.

Histological incision preparation and observation

Histology of rat liver and kidney was prepared using paraffin method and Hematoxylin-Eosin staining (Fisher *et al*, 2009). Observation of liver and kidney histology preparations of white rats was carried out at the Biosains Laboratory, Universitas Brawijaya using Image Raster application and OptiLab viewer application (Miconos, Jogjakarta, Indonesia). The histology of the liver and kidney of rats was observed by visual field observation and each preparation was observed as many as 5 fields of view. For one time, the visual field was counted for the number of normal liver and kidney cells and damaged cells such as necrosis, fatty degeneration, and hydropic degeneration in liver cells and fatty degeneration, necrosis and swelling of the glomeruli in the kidneys. After the results were obtained, the percentage of damaged cells was calculated for each field of view and the average percentage of cell damage was calculated for each treatment.

Study variables

The variables used in this study were control variables, independent variables and dependent variables. Control variable was CCl_4 1 ml/200g

body weight. The independent variables included cowpea tempeh extract with different doses in each group, namely 6% and 9%. The dependent variables included histology of the liver with types of damage such as necrosis, congestion, inflammatory cell infiltration, fatty degeneration, and hydropic degeneration and histology of the kidneys with damage such as fatty degeneration, necrosis, inflammatory cell infiltration, and glomerular swelling.

Data analysis

The data obtained from the observation of the histological incisions of the liver and kidneys were analyzed by statistical tests using the Statistical Package for the Social Sciences (SPSS) version 25 program (IBM, Chicago, IL). The normality of the data distribution was analyzed by the Saphiro-Wilk test. The normally distributed data were then analyzed using one-way analysis of variance (ANOVA). If there is a significant difference (p<0.05), then the analysis is continued with the Duncan's Multiple Range Test (DMRT).

RESULTS

This study investigated the effect of cowpea tempeh extract on the liver and kidneys of 28 male white rats (Rattus norvegicus) exposed by oxidative stress induced by carbon tetrachloride (CCl_4). Observation of liver and kidney histology preparations of study sample was prepared using paraffin method and Hematoxylin-Eosin staining.

Liver histology

Based on the results of observations on the histological incisions of the liver of white rats, it was found that there were damages in the form of fatty degeneration, hydropic degeneration, necrosis, inflammatory cell

infiltration, and congestion (Fig 1). The results of the analysis with the one-way ANOVA test showed a significant difference between the treatment and the control (p=0.043). To find out the location of the real difference between the treatment groups, it was continued with the DMRT test. The results of the analysis on the liver histology of white rats with hydropic degeneration showed that negative control (K(-)) group was significantly different from positive control (K(+)) and treatment group 1 (P1), but not significantly different from treatment group 2 (P2); K(+) was significantly different from P1 and P2; and P1 was significantly different from P2 (Table 1).

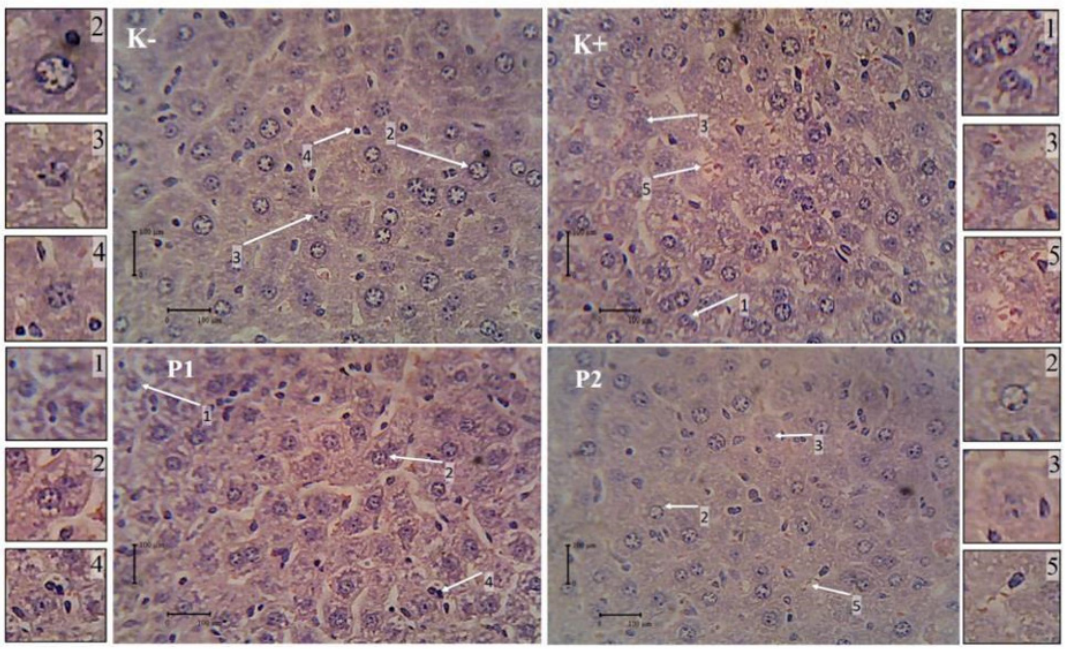

Fig 1 - Rat liver histology (400× magnification)

1: Fatty degeneration; 2: Hydropic degeneration; 3: Necrosis; 4: Inflammatory cell infiltration; 5: Congestion

K-: negative control; K+: positive control (induced by CCl_4 1 ml/200g body weight); P1: treatment group 1 (received CCl_4 1 ml/200g body weight and 6% tempeh extract; P2: treatment group 2 (received CCl_4 1 ml/200g body weight and 9% tempeh extract)

Kidney histology

Based on the results of histological incisions in the kidneys of white rats, damages found are fatty degeneration, necrosis, inflammatory cell infiltration, and swelling of the glomerulus (Fig 2). The results of the analysis showed a significant difference between the treatment and the control (p=0.032). The test results were then continued with the DMRT. The results showed that K(-) was significantly different from K(+), P1 and P2; K(+) was significantly different from P1 and P2; and P1 is significantly different from P2. The results of DMRT on kidney histology of rats with fatty degeneration, necrosis, inflammatory cell infiltration, and glomerular swelling showed that K(-) was significantly different from K(+), P1 and P2; K(+) was significantly different from P1 and P2; and P1 was significantly different from P2 (Table 2).

Table 1
Results of rat liver histology analysis

Variable and treatment	Mean ± standard deviation of rat liver histology
Fatty degeneration	
K-	1.69 ± 0.35
K+	12.88 ± 0.66^{c}
P1	11.02 ± 0.55^{b}
P2	6.47 ± 0.52^{a}
Hydropic degeneration	
K-	12.31 ± 1.21
K+	30.35 ± 1.65^{c}
P1	24.55 ± 1.15^{b}
P2	13.07 ± 1.67^{a}

Table 1 (cont)

Variable and treatment	Mean ± standard deviation of rat liver histology
Necrosis	
K-	20.55 ± 1.36
K+	61.33 ± 1.56[c]
P1	57.94 ± 1.60[b]
P2	35.38 ± 1.50[a]
Inflammatory cell infiltration	
K-	20.89 ± 1.90
K+	49.60 ± 1.80[c]
P1	39.41 ± 2.78[b]
P2	31.60 ± 2.26[a]
Congestion	
K-	16.76 ± 0.66
K+	48.37 ± 1.71[c]
P1	34.14 ± 1.31[b]
P2	26.69 ± 1.65[a]

Note: Analysis using one-way analysis of variance (ANOVA) followed by post-hoc Duncan's Multiple Range Test (DMRT). Numbers followed by different letters indicate statistically significant differences.

[a] $p<0.001$ when comparing with the negative control group (K-);
[b] $p<0.05$ when comparing with the positive control group (K+);
[c] $p<0.01$ when comparing with the positive control group (K+)

K-: negative control (rats in this group did not receive any treatment); K+: positive control (induced by carbon tetrachloride (CCl_4) 1 ml/200g body weight); P1: treatment group 1 (received CCl_4 1 ml/200g body weight and 6% tempeh extract; P2: treatment group 2 (received CCl_4 1 ml/200g body weight and 9% tempeh extract)

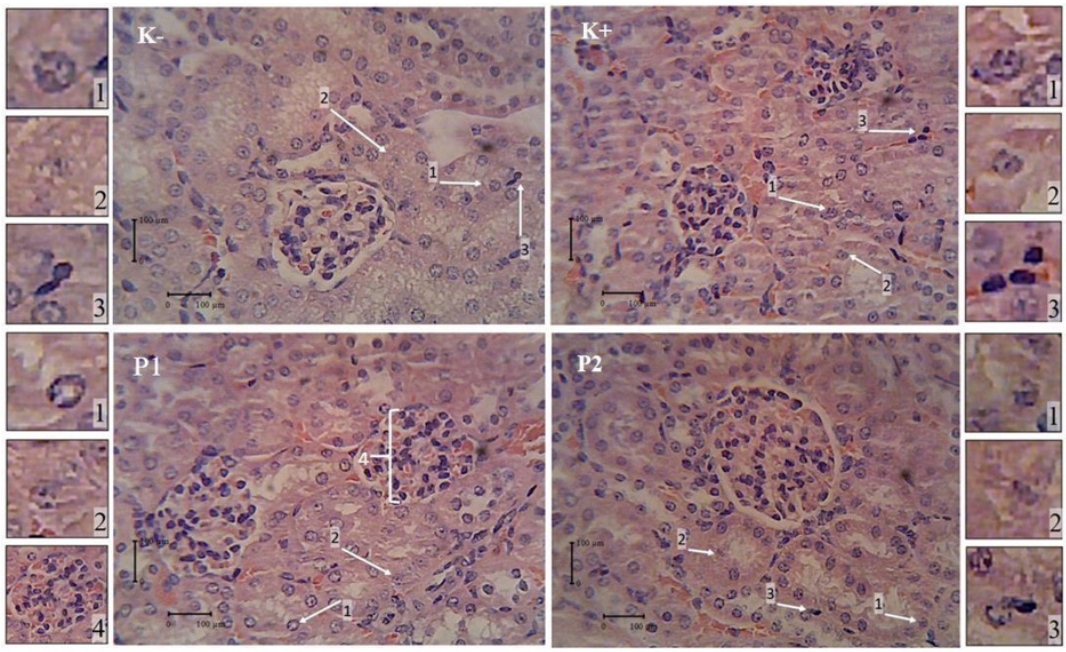

Fig 2 - Kidney histology in white rats (400× magnification)

1: Fatty degeneration; 2: Necrosis; 3: Inflammatory cell infiltration; 4: Glomerular swelling

K-: negative control; K+: positive control (induced by CCl_4 1 ml/200g body weight);
P1: treatment group 1 (received CCl_4 1 ml/200g body weight and 6% tempeh extract;
P2: treatment group 2 (received CCl_4 1 ml/200g body weight and 9% tempeh extract)

Table 2
Results of rat kidney histology analysis

Variable and treatment	Mean ± standard deviation of rat kidney histology
Fatty degeneration	
K-	2.60 ± 0.46
K+	8.87 ± 0.83^{c}
P1	7.57 ± 0.47^{b}
P2	6.71 ± 0.64^{a}

Table 2 (cont)

Variable and treatment	Mean ± standard deviation of rat kidney histology
Necrosis	
K-	8.60 ± 0.68
K+	26.43 ± 1.43[c]
P1	17.10 ± 0.88[b]
P2	14.41 ± 0.56[a]
Inflammatory cell infiltration	
K-	18.26 ± 1.62
K+	34.07 ± 1.52[c]
P1	28.46 ± 0.31[b]
P2	22.25 ± 0.62[a]
Glomerular swelling	
K-	24.38 ± 2.25
K+	48.95 ± 2.01[c]
P1	40.36 ± 2.09[b]
P2	33.60 ± 1.90[a]

Note: Analysis using one-way analysis of variance (ANOVA) followed by post-hoc Duncan's Multiple Range Test (DMRT). Numbers followed by different letters indicate statistically significant differences.

[a] $p<0.001$ when comparing with the negative control group (K-);
[b] $p<0.05$ when comparing with the positive control group (K+);
[c] $p<0.01$ when comparing with the positive control group (K+)

K-: negative control (rats in this group did not receive any treatment); K+: positive control (induced by carbon tetrachloride (CCl_4) 1 ml/200g body weight); P1: treatment group 1 (received CCl_4 1 ml/200g body weight and 6% tempeh extract; P2: treatment group 2 (received CCl_4 1 ml/200g body weight and 9% tempeh extract); SD: standard deviation

DISCUSSION

Liver histology

The results of observations made on histological incisions of the liver of white rats after being given treatment in the form of carbon tetrachloride at a dose of 1 ml/200 g body weight showed damages as fatty degeneration, hydropic degeneration, necrosis, inflammatory cell infiltration and congestion. Fatty degeneration (steatosis) is an abnormal accumulation of fat in the cytoplasm of cells caused by disorders of fat metabolism. The formation of fatty degeneration is caused by trichloromethyl free radicals (CCl_3) reacting with oxygen to form trichloromethyl peroxyl radicals which result in lipid peroxidation (Islam *et al*, 2017). This lipid peroxidation causes damage to the plasma membrane which can lead to the activation of a number of enzymes such as adenosine triphosphatase (ATPase). Activation of this enzyme causes a decrease in adenosine triphosphate (ATP) synthesis and resulting in a decrease in lipoprotein production so that lipid transport is disrupted and lipids will accumulate in hepatocytes (Suharyadi *et al*, 2014). According to Istikhomah and Lisdiana (2015), a decrease in ATP synthesis also results in a decrease in sodium pump activity so that the water outside the cell is attracted to the inside and the cell becomes swollen. Swelling of these cells results in hydropic degeneration.

Degenerated hepatocytes can return to normal if the damage-inducing agent is stopped, but if this situation continues it will result in necrosis. Panjaitan and Masriani (2014) stated that necrosis can occur because trichloromethyl peroxyl free radicals cause lipid peroxidation, thereby disrupting Ca2+ homeostasis. This causes intracellular hypercalcemia resulting in cell death (necrosis) of hepatocytes. Damage to cell membranes caused by free radicals also triggers an inflammatory response in the form of inflammatory cell infiltration. Inflammatory cell infiltration is a condition where inflammatory cells enter the tissue in response to a disease or toxic agent (Dinarello, 2010). Research Okolo *et*

al (2017), stated that the induction of CCl_4 compounds causes damage such as bile duct epithelial proliferation, sinusoidal dilatation and congestion.

Giving cowpea tempeh ethanol extract doses of 6% (P1) and 9% (P2) can cause a decrease in the level of liver cell damage caused by carbon tetrachloride. This is shown by the significantly decreased the percentage of cell damage observed in P1 and P2 compared to the positive control (K+). Hydropic degenerations in P2 were not significantly different from the negative control (K-). These results indicate that the administration of 9% cowpea tempeh extract can reduce the percentage of hydropic degeneration until it reaches a normal state. Kardena and Winaya (2011) stated that the initial type of degeneration that occurs in liver cells is hydropic degeneration. Degeneration is an early sign of cell damage that is temporary and can return to normal.

The decrease in cell damage can be caused by antioxidant compounds contained in cowpea tempeh extract such as flavonoids. Flavonoids are known to be able to donate hydrogen atoms to compounds that are free radicals so that these compounds become more stable. Saponins have the ability to reduce compounds that are free radicals through the formation of hydroperoxide intermediates that can prevent biomolecular damage in the body (Kartikasari *et al*, 2019). Research from Yuhernita and Juniarti (2011) also states that antioxidant compounds in the form of alkaloids contained in Surian leaves can stop the chain reaction of free radicals.

Kidney histology

Fatty degeneration is characterized by the discovery of fat-filled vacuoles in the cytoplasm and the nucleus being pushed to the edge. It was caused by lipid peroxidation which caused lysis of the cell membrane and was followed by mitochondrial damage, so that the cells were unable to eliminate water and triglycerides (Soepraptini *et al*, 2012). Necrosis was also found; it was caused by oxidation of lipids, proteins, carbohydrates,

and DNA by reactive oxygen species (ROS) which resulted in DNA mutations that resulted in cell death (Venkataarayana *et al*, 2012).

This study also showed the swelling of glomerulus, so that Bowman's space looked narrowed. Glomerular swelling can be caused by the entry of toxic substances into the glomerulus which results in increased capillary permeability and glomerular filtration, resulting in leakage of plasma proteins and red blood cells and resulting in swelling of the glomerulus (Mayori *et al*, 2013).

The results of the calculation of the data obtained in the histological incision of the kidney after administration of cowpea tempeh ethanol extract doses of 6% (P1) and 9% (P2) showed a significant reduction in fatty degeneration, necrosis, inflammatory cell infiltration and glomerular swelling compared with positive control (K+). This decrease in cell damage can be caused by antioxidant compounds in the form of flavonoids found in cowpea tempeh extract. The results of this study are supported by the research of Al Idrus *et al* (2014) who stated that antioxidants in the form of alkaloids, flavonoids, saponins, phenolics, steroids and terpenoids in the leaf extract of Mecca can reduce the degree of lung damage exposed to free radicals due to cigarette smoke.

In summary, cowpea tempeh ethanol extract doses of 6 % (P1) and 9% (P2) can repair liver and kidney damage in white rats after being induced by carbon tetrachloride (CCl_4), with a class of chemical compounds contained in cowpea tempeh extract such as flavonoids. Cowpea tempeh could be served as an alternative functional food that rich in antioxidants to prevent oxidative stress that can damage the human body.

ACKNOWLEDGMENTS

We really appreciated to those who have participate in providing support in terms of knowledge, material, enthusiasm, and others.

First to the Board of Research and Community Service (Badan Penelitian dan Pengabdian Masyarakat), Faculty of Health Sciences, Universitas Brawijaya for the opportunity given as well for the support, knowledge, and facilities provided to conduct this study in 2022.

This study was funded by Non-tax Revenue (Penerimaan Negara Bukan Pajak) 2022 Faculty of Health Sciences, Universitas Brawijaya with Main Researcher Grant (Hibah Peneliti Utama) scheme.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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