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Effect of Chronic Toxicity Studies of Sappan Wood Extract on The Kupffer Cells Number in Rats (*Rattus novergicus*)

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ABSTRACT

Sappan wood (*Caesalpinia sappan* L.) is a plant used by the community to mix drinking. The antioxidant index of Sappan wood extract has a higher value than commercial antioxidants to counteract oxidative free radicals and improve the body's defense system. The use of Sappan wood drinking in society is often used in daily so it is necessary to do a chronic toxicity test to observe the response to prolonged use of the system in the body. Kupffer cells are an important part of the innate immune system, acting as "scavengers" and phagocytes. A study that aims to see the chronic toxicity studies of the ethanolic extract of Sappan wood on the number of Kuffer cells in the liver has been carried out on 60 Wistar rats divided into 12 groups. The group was the negative control group, doses 100 mg/kgBW, 200 mg/kgBW, 300 mg/kgBW, 400 mg/kgBW and 500 mg/kgBW of extract Sappan wood which were divided into male and female groups. Each group was given ethanol extract of Sappan wood for one year orally. The results showed a significant increase in Kupffer cells in the female group at a dose of 100 mg/kgBW and the male group at 200 mg/kgBW with significant values, respectively, $p<0.001$ $p = 0.004$.

1. Introduction

Sappan wood (*Caesalpinia sappan* L.) is one of the plants used as traditional medicine. Sappan is a herbal plant that grows naturally in secondary forests. Empirical use of Sappan wood is not only used to improve health, but it can also be used for various treatments, such as wound healing to cancer (Suyatmi *et al.* 2019). Sappan wood contains phenolic compounds such as flavonoids, xanthones, coumarins, chalcones, flavones, isoflavonoids, and brazilin (Prashith *et al.* 2019). Phytochemical tests showed that Sappan wood contains alkaloids, flavonoids, phenols, and saponins. The results of the isolation of phytochemical compounds in several studies state that those that act as antioxidants in Sappan wood are brazilin and flavonoids (Sufiana and Harlia 2014).

Several researchers have conducted several studies to increase the immune system using Sappan wood. Research by Sunitha *et al.* (2015) showed that the ethanolic extract of Sappan wood at a dose of 25 mg/kgBW showed a significant increase in phagocytic activity ($p<0.05$) compared to controls. Peritoneal macrophages showed that research increased phagocytic response after *in vitro* administration of the ethanolic extract of Sappan wood. This study demonstrated the immunomodulatory effect of Sappan wood extract on murine peritoneal macrophages, which is a non-specific immune mechanism.

The immune system can be affected by feed, pharmacological agents, environmental pollution, and natural chemicals (natural substances) such as vitamins and flavonoids. The plant contains a flavonoid useful in increasing red blood cell production, growth hormone production, stimulating liver function in neutralizing toxins, and increasing the body's defenses by inducing cellular immunity to fight infectious and bacterial diseases giving that medicinal herb which flavonoid component is expected to be able to improve the body's defense system, especially in increasing cellular immune responses. One organ that plays a role in the body's defense system is the liver (Panche *et al.* 2016; Slevin *et al.* 2020).

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The liver is the largest digestive gland and the most complex metabolic center in the body. Besides metabolism, the liver is also a storage place for nutrients absorbed from the digestive tract and the main biotransformation organ for further use by other body parts (Maulina 2018). The liver also has a role in the body's defense system because the liver has the Kupffer cells that play for phagocytosis and immunity (Astuti *et al.* 2020). Kupffer cells are hepatic macrophages and play an important role in maintaining for liver function. Under physiological in normal conditions, the Kupffer cell is the first innate immune cells which serves to protect against microorganisms infection homeostasis, and participate in the acute and chronic responses of the liver to toxic compounds (Roberts *et al.* 2007; Tsutsui and Nishiguchi 2014; Nguyen-Lefebvre and Horuzsko 2015). The role of Kupffer cells in the sinusoids of the liver is to perform the general function of tissue macrophages, including the response to tissue damage and antigen presentation. Kupffer cells are also involved in specialized activities, including iron and particles uptake from portal blood (Bennett *et al.* 2021). Seeing the importance of Kupffer cells in the body's defense system, this study was conducted to count the changes in the number of Kupffer cells in rats given ethanol extract of Sappan wood with variations of dosage for one year as the basis for chronic toxicity tests. By looking at the number of Kupffer cells, it can be seen how the role of Sappan wood in increasing the immune system in the body.

2. Materials and Methods

2.1. Production of Extract of Sappan Wood (*Caesalpinia sappan* L.)

Sappan wood powder was weighed as much as 1 kg and macerated using 95% ethanol as much as 1 L at room temperature, after 24 hours, the supernatant was taken and stored separately. The remaining residue was then added with 96% ethanol and soaked again for 24 hours. The same procedure was repeated until 4 liters of 96% ethanol were used for 1 kg of Sappan wood powder. All the collected filtrate was then evaporated using a rotary evaporator at a temperature of 40-60°C until a dry extract was obtained.

2.2. Animal Treatment

Male and female rats were separated into six groups which were utterly randomized for each sex. Each group was acclimatized in the laboratory for seven days. After the acclimatization stage was completed, the rats were given Sappan wood extract dissolved in distilled water with the addition of 0.5% CMC. Sappan wood extract solution was carried out orally with variation dose adjusted for 1 ml every 300 grams of rats. Sappan wood extract was given every 24 hours for 12 months. Sappan wood extract was given in different doses are normal group (without Sappan wood), 100 mg/kgBW, 200 mg/kgBW, 300 mg/kgBW, 400 mg/kgBW, and 500 mg/kgBW.

2.3. Histological Examination

On the last day of treatment, the animals were anesthetized using ketamine xylazine, and their livers were removed for slide histological. The liver is processed with paraffinization with the routine procedure and stained with Hematoxylin Eosin. The collected liver was soaked with 10% neutral buffer formalin and continued with the process of dehydration, clearing, infiltration, and embedding. The resulting tissue blocks were then cut using a microtome thickness of 5 µm and observed by light microscope Olympus CX-23® (Khristian 2021).

2.4. Data Analysis

Data was collected from slide histology with a total magnification of 400x. Fields of view were taken randomly as many as five fields of view in the periportal area. Kupffer cells that are visible in the field are counted using the ImageJ software as a marker for each cell that has been measured. Kupffer cells observed then calculated using the "cell counter" plugin in ImageJ software. The calculation results were then analyzed with ANOVA and Duncan's post hoc test using SPSS 26 software.

3. Results

3.1. Histological of Hepatocytes dan Kupffer Cells in the Male Rat

The results of Kupffer cell morphology among liver hepatocytes on male rats are shown in Figure 1.

The results showed that the distribution of Kupffer cells between the sinusoidal spaces was evenly distributed. The hepatocytes show normal morphology cells without significant damage. The results of the calculation cells number found are shown in Table 1. Hepatocyte cells show a nucleus that is clearly visible chromatin granules without any signs of damage or accumulation of nuclear components that allow necrosis or hyperchromatism. Hepatocyte cytoplasm generally normal without any signs of swelling or vacuolization.

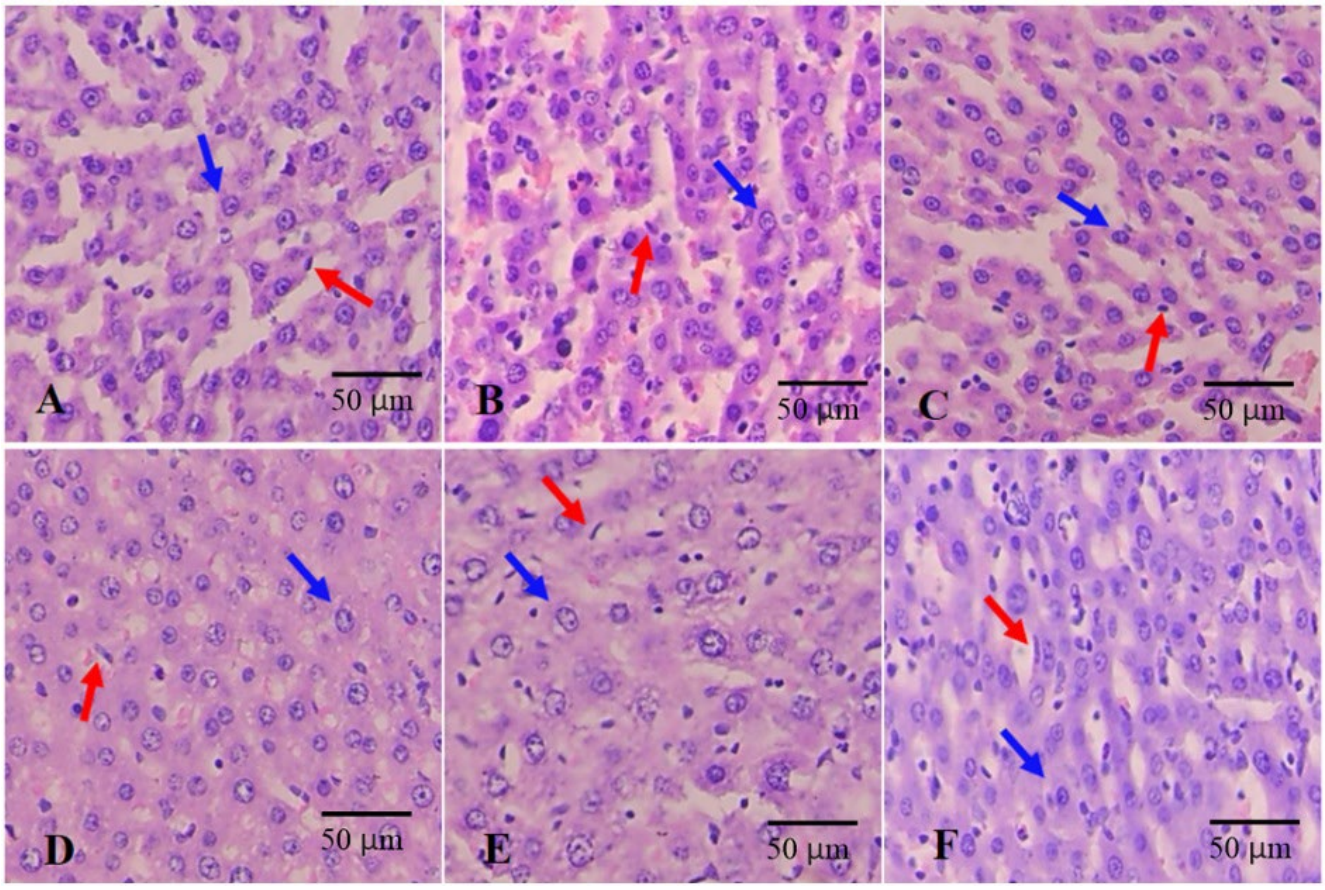

Figure 1. Morphology of hepatocytes and Kupffer cell of male rats' livers stained with Hematoxylin Eosin staining and observation using 400x magnification. The pictures shown of the control group (A), dose of 100 mg/kgBW (B), dose of 200 mg/kgBW (C), dose of 300 mg/kgBW (D), dose of 400 mg/kgBW (E), dose of 500 mg/kgBW (F). Blue arrows indicate hepatocytes, and red arrows indicate Kupffer cells

Table 1. Data analysis of Kupffer cell number

Groups	N	ANOVA p-value male/female	Male	Female
			Mean±SD	Mean±SD
Kontrol negatif	5	<0.001/<0.001	53.2±8.7^{ab}	31±9.3^{a}
Dosis 100 mg/kgBB	5		45.8±3.7^{a}	56.6±6.4^{b}
Dosis 200 kg/kgBB	5		52.4±8.0^{ab}	53±6.3^{b}
Dosis 300 kg/kgBB	5		59.4±4.0^{bc}	54.8±4.8^{b}
Dosis 400 mg/kgBB	5		64.4±1.7^{c}	54.8±2.6^{b}
Dosis 500 mg/kgBB	5		76.8±2.6^{d}	68±5.7^{c}

3.2. Histological of Hepatocytes dan Kupffer Cells in the Male Rat

The results of Kupffer cell morphology among liver hepatocytes on female rats are shown in Figure 2.

The results showed that the distribution of Kupffer cells in each study group was evenly distributed between the sinusoidal spaces. Same as in the female group of mice, the hepatocyte cells appeared normal without any signs of damage or cell death. Swelling and vacuolation of the cytoplasm was not seen.

3.3. Statistical Analysis of Kupffer Cell Number

After histology observation, quantitative analysis was carried out by counting the number of Kupffer cells. The results of the calculation of the number of Kupffer cells are shown in Table 1 below. The results of the data analysis of Kupffer cells both descriptive and mean different analysis shown in Table 1.

The average of Kupffer cell number ranges from 45.8 to 76.8 cells for male rat and ranges from 31 to 68 cells for female rat. The data showed that there was an increase in the number of Kupffer cells in

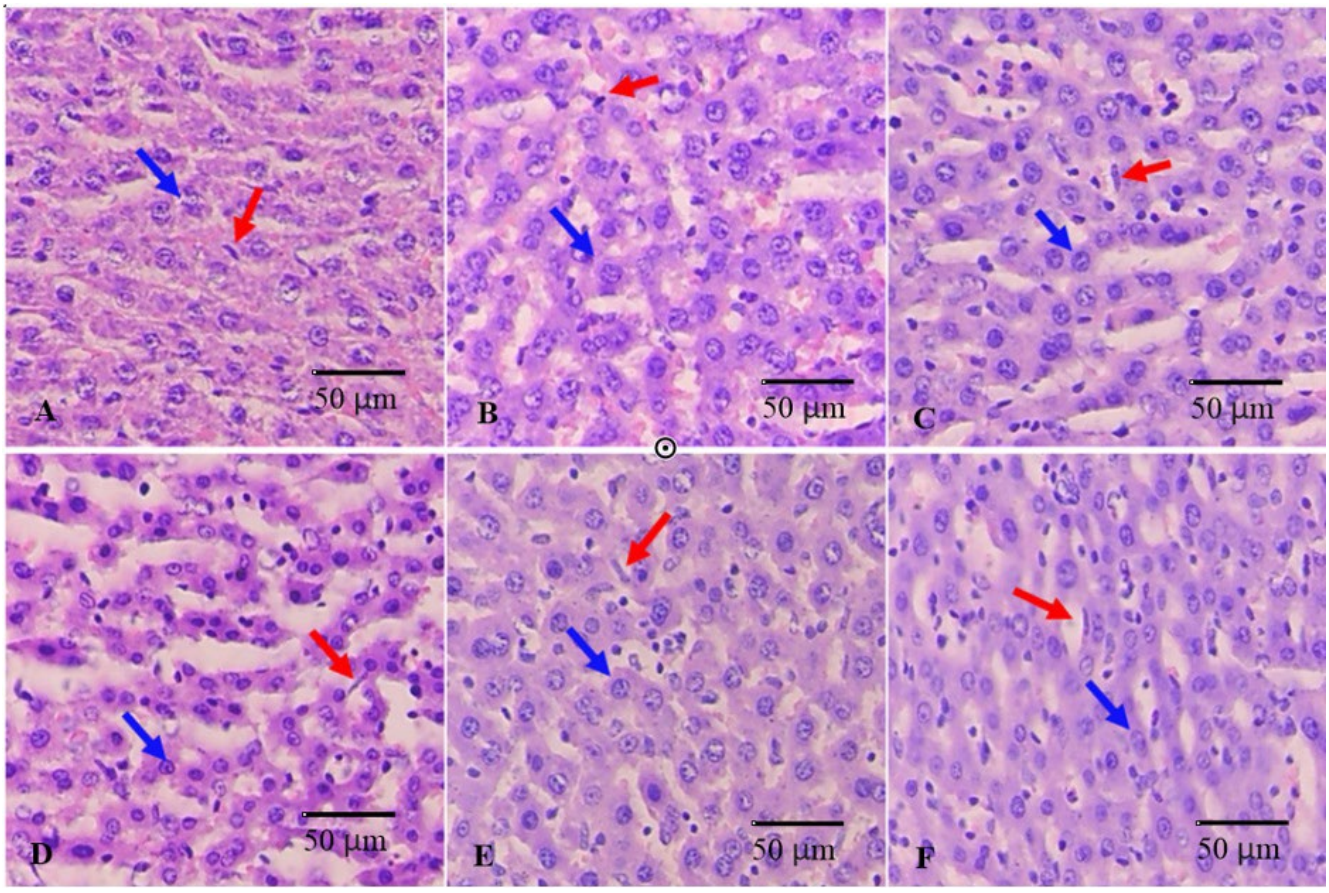

Figure 2. Morphology of hepatocytes and Kupffer cell of female rats' livers stained with Hematoxylin Eosin staining and observation using 400x magnification. The pictures are control group (A), dose of 100 mg/kgBW (B), dose of 200 mg/kgBW (C), dose of 300 mg/kgBW (D), dose of 400 mg/kgBW (E), dose of 500 mg/kgBW (F). Blue arrows indicate hepatocytes, and red arrows indicate Kupffer cells

both male and female groups along with the increase in dose. In the male group, there were unique data, where in the group that was given of sappan wood extract at a dose of 100 mg/kgBW experienced a decrease. In the female group, doses of 300 mg/kgBW and 400 had the same mean value even though the SD values were different.

The analysis results with the ANOVA tests for male and female showed p-values are <0.001, so it can be said that there has been a significant difference between groups (95% confidence level). The results of Duncan's analysis showed that the group of male rats given Sappan wood extract at a dose of 100 mg/kgBW and 200 mg/kgBW was not significantly different compared to the negative control group. The negative control group has started to show a significant difference compared to the male rat's given Sappan wood extract at a dose of 400 mg/kgBW and 500 mg/kgBW. The administration of the highest amount of 500 mg/kgBW is the most different group than other groups. This analysis showed a significant increase in the number of Kupffer cells in the group of male rats given sappan wood extract at doses of 400 mg/kgBW and 500 mg/kgBW.

The results of Duncan's analysis for the female rat showed that the administration of Sappan wood extract at 100 mg/kgBW was significantly different when compared to the control. Doses of 100 mg/kgBW, 200 mg/kgBW, 300 mg/kgBW, and 400 mg/kgBW had the same effect in increasing the number of Kupffer cells. The dose of 500 mg/kgBW had a significantly different value compared to the control group and the group given other doses of Sappan wood extract.

4. Discussion

The results of the study for all groups with different doses in both male and female showed normal hepatocyte. Normal hepatocytes were seen as cells with a well-defined nucleus, without vacuolization, clear, and normochromatic nuclei. Central veins and

hepatic sinusoids still looks white (normal) which indicates that there is no congestion. In the control group, both male and female gave a normal picture with a small number of some degenerative changes such as the presence of hepatocyte cell nuclei that experienced cloudy swelling and even necrosis of cells. Changes in cell degeneration and cell death in the control group may be caused by several physiological factors, environmental conditions that cannot be controlled during the study, or natural conditions (Kumar *et al.* 2020). In the group presenting the Sappan wood extract, it showed the same thing which generally looked normal but there were some cells that underwent degenerating changes to necrosis. The changes still showed normal values that were comparable to the negative control group, both for male and female sexes.

Under normal circumstances, Kupffer cells play an important role in maintaining immune tolerance of the liver (these cells are in a permanent semi-active state mainly due to continuous exposure to antigens that reach the organ from the gut). Kupffer cells can release tumor necrosis factor-alpha (TNF-a), interleukin (IL)-6, IL-1b, or leukotrienes, which attract T cells and induce hepatocyte apoptosis hepatic stellate cell activation. Kupffer cells are highly involved in the liver's response to various toxic disorders. The interaction of Kupffer cells with leukocytes is important for the defense of liver cells from all disturbances, microorganisms, chemical compounds, or other solid materials (Bilzer *et al.* 2006).

The study results show an increase in Kupffer cells for both male and female rats. In the male group, a significant increase was seen starting at a dose of 300 mg/kgBW for male rat, and a dose of 100 mg/kgBW for female rat. In the male rat group, a significant increase continued at doses of 400 mg/kgBW and 500 mg/kgBW, where at a dose of 500 mg/kgBW was the dose that produced the highest number of Kupffer cells. In contrast to the female rat group where a significant increase occurred again at a dose of 500 mg/kgBW.

This increase in Kupffer cells becomes very important under certain conditions because Kupffer cells can play a role in guarding, monitoring, and cleaning unwanted particles (Woltman *et al.* 2014). The increase in Kupffer cells in liver cells can be caused by several factors such as the presence of antigens, foreign particles such as metals, and compounds that can stimulate the addition of Kupffer cells. Kupffer cells in the liver are used for protection in several situations, including drug-induced liver injury and toxic fibrosis. Kupffer cells are upregulated in the precise control of the inflammatory response contributing to chronic inflammation in the liver. Other evidence suggests that Kupffer cells play critical protective functions in hepatocyte proliferation in response to hepatotoxic injury, as well as in the resolution of fibrotic scar tissue (Ramachandran and Iredale 2012; Dixon *et al.* 2013). The increase in Kupffer cells in various studies has been suggested to have an important protective function in the liver by producing various modulating factors that can counteract the inflammatory response and stimulate liver regeneration (Ju *et al.* 2002).

The significant increase in the number of Kupffer cells from the administration of extract Sappan wood on the results indicated the role of the active compounds in the extract that influenced it. Compounds found in Sappan wood extract include homoisoflavonoids and polyphenols (Syamsunarno *et al.* 2021). The part of the homoisoflavonoid compound from the section of Sappan wood used as the main component and has pharmacological properties is brazilin (Sufiana and Harlia 2014; Prashith *et al.* 2019).

The increase in the number of Kupffer cells in this study could be due to polyphenol compounds in the Sappan wood extract. Several articles show that the effect of polyphenols can function as an immune system enhancer in the body. Each type of polyphenol targets and binds to one or more receptors on immune cells and triggers intracellular signaling pathways that ultimately regulate the immune response. Administration of polyphenols can modulate immune responses by influencing epigenetic mechanisms, such as regulation of DNA methylation, histone modification, and microRNA-mediated post-transcriptional repression (Ding *et al.* 2018).

Other compounds that were allegedly able to increase Kupffer cells in this study were flavonoids. A research review from Pan *et al.* (2020) stated that flavonoids in plants could activate immune cells, especially Kupffer cells in the liver. The increase in Kupffer cells in rats given the extract of Sappan wood is beneficial for increasing the immune system in the body and can be helpful in the performance of liver cells and their metabolism (Susanto *et al.* 2014). This follows the research of Hassan *et al.* (2020), which states that an increase in Kupffer cells in an appropriate number can increase the metabolic system. The normal morphology of liver cells also indicates this from the entire field of view obtained or around the observed Kupffer cells.

The number of Kupffer cells which was increased by increasing the doses of Sappan wood extract

indicated that there were components that were able to increase the growth of Kupffer cells. The increase in the number of Kupffer cells indicates that the use of Sappan wood extract can improve the immune system in the body. Increased Kupffer cells will produce inflammatory cytokines that can trigger oxidation reactions or free radicals from compounds that enter the body, especially the liver (Slevin *et al.* 2020; Bennett *et al.* 2021).

In conclusion, the results of the chronic toxicity study of Sappan wood extract in male and female rats showed a significant effect on increasing the number of Kupffer cells.

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