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HISTOPATHOLOGICAL STRUCTURE OF THE RAT KIDNEY AFTER ADMINISTRATION OF SAPPAN WOOD EXTRACT (*Caesalpinia sappan L.*) IN IRON OVERLOAD CONDITION

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Abstrak

Jumlah zat besi yang berlebih dalam tubuh dapat memicu kerusakan pada berbagai organ termasuk ginjal. Penggunaan kelator besi telah terbukti dapat mengurangi akumulasi zat besi. Penelitian ini bertujuan untuk mengetahui pengaruh pemberian ekstrak kayu secang (Caesalpinia sappan) sebagai adjuvant kelator besi terhadap serta struktur dan fungsi ginjal tikus model besi berlebih. Penelitian ini dilakukan secara eksperimental selama 28 hari dengan Rancangan Acak Lengkap (RAL) yang terdiri atas 6 kelompok perlakuan. Iron dextran 60 mg/kg BB diberikan agar terjadi kondisi besi berlebih. Deferiprone 1,35 mg/kg BB diberikan sebagai kelator besi pembanding. Ekstrak kayu secang (EKS) diberikan pada tiap kelompok uji dengan dosis 100, 150, dan 200 mg/kg bb. Parameter yang diamati meliputi pemeriksaan distribusi besi dan pengamatan struktur kerusakan ginjal. Data yang diperoleh dianalisis dengan menggunakan analisis varian (ANOVA) pada taraf kepercayaan 95% dan dilanjutkan dengan uji beda Duncan. Hasil penelitian menunjukan bahwa terdapat perbedaan kadar besi ginjal pada setiap kelompok perlakuan ($p < 0.05$) terhadap kelompok kontrol besi berlebih. Hasil pemeriksaan histopatologis menunjukan pemberian ekstrak kayu secang memiliki perbedaan skor kerusakan terhadap kelompok kontrol besei berlebih ($p < 0.05$). pemberian EKS dosis 100 mampu menurunkan kadar besi dan mengurangi kerusakan pada organ ginjal tikus model besi berlebih.

***Kata Kunci**: Kayu Secang (Caesalpinia sappan L), Besi Berlebih, Ginjal*

Abstract

Excessive amounts of iron in the body can lead to damage to various organs, including the kidneys. Iron chelators have been demonstrated to effectively reduce the accumulation of this excess iron. This study aims to investigate the impact of administering sappan wood extract (Caesalpinia sappan) as an adjuvant to iron chelators on the structure and function of kidneys in a rat model of iron overload. The experimental research, spanning 28 days, employed a Completely Randomized Design (CRD) involving 30 male Wistar rats (Rattus norvegicus) distributed across 6 test groups. Iron dextran at 60 mg/kg bb induced iron overload, while a comparative iron chelator, deferiprone, was given at 1.35 mg/kg bb. Various doses of Sappan wood extract (SWE) 100, 150, and 200 mg/kg bb were administered to separate test groups. The parameters that are being observed include the distribution of iron and the structure of kidney injury. At a 95% confidence level, the acquired data were examined using analysis of variance (ANOVA), and the Duncan test was used to see whether there were any differences. The study's findings demonstrated that each treatment group's kidney iron levels differed from the excess iron-containing control group ($p < 0.05$). The

histological investigation results demonstrated a significant difference in damage scores ($p < 0.05$) between the groups administered sappan wood extract and the excess base control group.
Adminstered of 100 mg/kgbw dose of EKS might lower their organ iron levels and lessen the harm that the iron did to their kidneys.
Keyword: Sappan Wood (Caesalpinia sappan L.), Iron Overload, Kidney

1. INTRODUCTION

The components of iron in the body are divided into three compartments: functional/essential iron, iron in transport (Transferrin), and iron reserves (Ferritin). Functional/essential iron includes iron in hemoglobin or heme iron (70%), iron in oxygen-binding proteins (TIBC: Total Iron Binding Capacity), iron found in muscles (myoglobin) at 4%, and iron in enzymes such as cytochrome, catalase, peroxidase (<1%). Iron in transport (Transferrin) is present in small amounts in the blood and is in the form of ferrous iron (Fe2+).

Iron usually enters the body through the digestive tract and is absorbed in the duodenal enterocytes (Seyoum et al., 2021). However, in some cases, such as patients with Thalassemia major, iron can be obtained through routine blood transfusions. Thalassemia is an autosomal recessive inherited blood disorder caused by a decrease or absence of globin chain synthesis, leading to chronic hemolytic anemia and ineffective erythropoiesis, requiring lifelong periodic blood transfusions to maintain hemoglobin levels. This can lead to an excess of iron in the body (Bajwa & Basit, 2022).

Excess iron plays a significant role in oxidative stress. When there is an excess of iron in the body, plasma transferrin becomes saturated, unable to bind excess iron, and this iron becomes a non-transferrin-bound iron (NTBI) (Long et al., 2023). NTBI can be filtered along with the blood into the kidney tubules and trigger damage to the kidney cells. According to Sadeghi et al. (2021), the effect of thalassemia on the kidneys has not been extensively evaluated. However, up to 60% of patients among 25,000 individuals with beta-thalassemia major have been reported to exhibit signs of tubular dysfunction, primarily affecting the proximal tubules. According to Yurt et al. (2013), damage to the proximal tubules can be marked by necrosis, lipid degeneration, and hydropic degeneration caused by various factors. According to Verdiansah (2016), measuring serum urea is performed to help diagnose acute kidney failure. The National Kidney Disease Education Program also recommends using serum creatinine to measure glomerular filtration rate, which is used to monitor the course of kidney disease. The diagnosis of kidney failure can be made when the levels of creatinine and urea in the serum rise above normal reference values.

The use of iron chelators has been proven effective in reducing iron accumulation in patients who receive regular blood transfusions, such as thalassemia patients. Deferiprone is commonly used as an iron chelator because it can be administered orally with a dose of 25 mg/kg body weight three times a day (75 mg/kg body weight/day) (Entezari et al., 2022). Deferiprone has been shown to significantly reduce iron deposition in the glomerulus, interstitial zone, and proximal tubules in iron-overloaded rat models and thalassemia rat models (Yatmark et al., 2016). However, according to Morales et al. (2016), deferiprone administration did not significantly reduce serum NTBI levels, so an adjunct iron chelator is needed to enhance the effectiveness of deferiprone. Additionally, Arya et al. (2020) suggest that long-term use of deferiprone can lead to various side effects such as agranulocytosis and neutropenia.

Sappan (Caesalpinia sappan, L.) is a plant containing various antioxidant compounds. According to Safitri et al. (2018), active antioxidant compounds from the stem of Sappan wood (C. sappan L.), such as brazilin and flavonoids, can neutralize superoxide anion radicals,

scavenge hydroxyl free radicals, and chelate iron ions. Safitri et al. (2016) also stated in their research that the effectiveness of an iron chelator depends on its ability to bind free iron that is not bound to transferrin and circulates in the plasma. Administration of Sappan wood extract at a dose of 100 mg/kg body weight increased transferrin levels. High transferrin levels indicate a reduction in free iron in the plasma. According to Jia et al. (2016), brazilin in Sappan can provide protective effects on the kidneys of rats with acute kidney injury.

Based on previous research, it can be concluded that Sappan wood extract has the potential as an iron chelator and can be protective of the kidneys. However, the effective dose of Sappan wood extract combined with deferiprone as an adjuvant to prevent damage to various essential organs, especially the kidneys, is not yet known. According to Johnston et al. (2011), adjuvant therapy is used to enhance the effectiveness of the main drug when used concurrently with additional drugs. Therefore, the parameters observed in this study include kidney organ iron levels, as well as the histological and physiological structure of the kidneys. Histological observations include assessing damage to kidney cells characterized by necrosis, as well as the observation of cells undergoing lipid degeneration and hydropic degeneration. The parameters for kidney function assessment include urea and creatinine levels in the rat's blood serum.

2. RESEARCH METHOD

This research was conducted experimentally in a laboratory using the Completely Randomized Design method with 6 treatments with 5 rats in each group. The treatment groups included the control group (only given free access to food and water), negative control group (Iron Dextran (ID) 60 mg/kg BW), positive control group (ID 60 mg/kg BW and Deferiprone (DFP) 75 mg/kg BW), and various doses of Sappan wood extract 100 mg/kg BW (A1), 150 mg/kg BW (A2), and 200 mg/kg BW (A3). The treatments were administered orally for Deferiprone and Sappan wood extract daily for 28 days, while ID was injected intravenously for 14 days with a 3-day interval. Subsequently, all rats underwent surgery on the 29th day after fasting for 16 hours. This study has been approved and registered with the Research Ethics Commission of Padjadjaran University under Ethical clearance Number 605/UN6.KEP/EC/2021.

The equipment used in this research included tools for the maintenance and treatment of test animals, tools for organ isolation, as well as tools for observing functional and histological parameters. The materials used included test animals, test substances, and chemicals. The test animals used were male Wistar strain rats (Rattus norvegicus L.) aged 8 weeks with an average body weight of 200 grams and rat pellet feed CP-551. The test substances used were Sappan wood extract (Caesalpinia sappan L.), Iron Dextran, and Deferiprone. The chemicals used included alcohol (70%, 80%, 90%, 95%), 1% acetic acid, hematoxylin Weigert [Merck®], entelan [Merck®], eosin [Merck®], ethanol (70%, 80%, 90%) [Merck®], absolute ethanol [Merck®], hematoxylin [Merck®], Ketamine xylazine, xylene solution, liquid paraffin pastilles [Merck®], creatinine reagent [Meril Creatinine Kit], and urea reagent [Meril Urea BUN Kit].

2.1 Preparation of Sappan Wood Extract

Sappan wood is shaved, and its bark is removed. The inner part of the Sappan wood is then processed into a powdered form, resulting in coarse powdered simplicia. The simplicity of Sappan is subjected to extraction using the maceration method with 96% ethanol solvent for four cycles of 24 hours each, resulting in a yellow-colored extract. Approximately 3 cm above the extract sediment is filled with 96% ethanol. The obtained liquid extract is then evaporated using a rotary evaporator at a temperature of 60°C until a dry extract is obtained (Sari et al., 2018).

2.2 Acclimatization, Maintenance, and Treatment

The test animals were acclimatized for seven days before treatment. Acclimatization was carried out to allow the rats to become accustomed to the laboratory environment. The rats were kept in a room with a temperature of approximately 26°C, with a 12-hour light and 12-hour dark cycle. The rat cages were in the form of animal housing boxes measuring 40 x 30 x 15 cm, covered with iron grilles, and lined with bedding. The cage bedding was replaced, and body weights were measured every three days, once a day. During acclimatization, the rats were fed CP-551 type feed and provided with water. At this stage, body weights were measured, and the rats were grouped based on treatment groups. The administration of Sappan wood extract was carried out daily for 28 days, administered orally using a gavage needle (BPOM, 2014 with modifications).

2.3 Measurement of Iron Levels in Kidney Organs

The iron content in the organs is measured using the Atomic Absorption Spectrophotometry (AAS) method with an AAS instrument of the AAnalyst 400 brand. To perform the measurement, the previously isolated organs are first weighed, approximately 0.2 g, and then placed in a 100 ml chemical glass. Next, 5 ml of HNO3 is added to the chemical glass, and it is heated to dryness. Afterward, 2 ml of 30% H2O2 is added to the chemical glass, and it is heated again until gas bubbles are observed. The solution is then further diluted to a volume of 25 ml in a measuring flask and shaken until homogeneous. The solution is ready to be measured using AAS with a wavelength of 248.3 nm. The iron content in the organs is then calculated using the following formula:

$$\text{Iron all } \frac{(C-B) \times V}{W} \text{ ppm}$$

note:

- C: The concentration that the device reads
- B: Blanko
- V: Volume
- W: Sample Weight

2.4 Kidney Function Examination

Blood was collected through the rat's heart using a syringe. The collected blood was transferred to a vacutainer tube, approximately 4 ml in volume. The collected blood was then centrifuged at a speed of 3000 rpm for 10 minutes. The serum was extracted and placed in a new Eppendorf tube. The tube containing the serum was then stored in a box or container with a cold temperature (containing ice) or directly placed in a refrigerator at -20ºC until the serum was to be used or tested (BPOM, 2014). The measurement of urea and creatinine levels was conducted using a spectrophotometer.

2.5 Hematoxylin-Eosin staining

Histology preparation is carried out by sectioning the specimens using a rotary microtome with a thickness of 5 µm, followed by staining with hematoxylin-eosin (HE) (Fahrimal et al., 2016). Firstly, staining is performed on a staining rack by immersing the specimens in the first xylene for 5 minutes and then dipping them into the second xylene 20 times. Afterward, the prepared specimens are sequentially dipped into ethanol 1, ethanol 2, 90% ethanol, 80% ethanol, and 70% ethanol, each for 20 dips. Next, the prepared specimens are rinsed with running water. The specimens are stained with hematoxylin for 5 minutes, then rinsed again with running water for 5 minutes. Next, the prepared specimens are dipped into 0.25% lithium carbonate four times and then rinsed again with running water. Subsequently, the specimens are dipped into 70% ethanol for 20 dips and stained with eosin for 20 seconds, followed by rinsing with running water. Next, a gradual dehydration process is carried out using 70%, 80%, and 90% ethanol for 20 dips each, and the specimens are dried in an incubator for 5 minutes. After that, the prepared specimens are immersed in xylene (2 times) for 20 dips each. Finally, the prepared specimens are treated with Entelan and covered with a glass coverslip (Yulianti, 2017).

The observed parameters include the percentage of cells undergoing necrosis, fat

degeneration, and hydropic degeneration, each of which is observed and counted in a 5 x 5 mm field of view, in 5 fields of view with a magnification of 400x (Modified from Fahrimal et al., 2016).

3. RESULTS AND DISCUSSION

3.1 Kidney Iron Levels

The kidneys of rats with iron overload models were examined for iron levels using the Atomic Absorption Spectroscopy (AAS) method. Additionally, the impact of providing sappan wood extract as an iron chelator adjuvant in conjunction with deferiprone was examined. An extremely precise and sensitive spectrophotometric analysis method for determining metal concentrations, including iron, is the AAS method. The analysis's findings demonstrated that in comparison to the normal control group, the kidneys of the rat in the iron excess model group had noticeably higher levels of iron. Figure 1 shows the average findings of the analysis of the iron levels in the renal organs of rats. One-way ANOVA analysis's findings indicate that there are notable variations in the treatment groups given (Sig.<0.05)

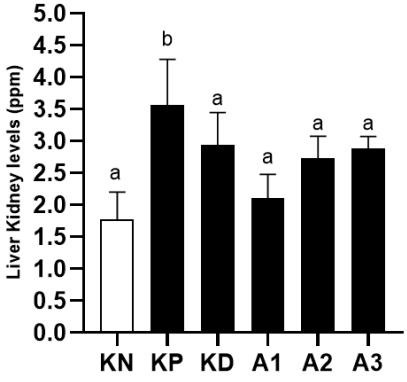

Figure 1. Level of iron level in kidney (KN) Normal Group; (KP) ID group; (KD) DFP + ID ; (A1) SWE 100 mg/kgBW; (A2) SWE 150 mg/kgBW; (A3) SWE 200 mg/kgBW Different letters (a, b, c) indicate significant differences among treatment groups based on the Duncan test ($p < 0.05$).

The kidney iron levels of the negative control group, which received iron dextran 60 mg/kg bw, were higher than those of the normal control group and the group that received sappan wood extract as an adjuvant for iron chelation, as shown by the diagram in Figure 1. This suggests that administering 60 mg/kg bw of iron dextran can affect and raise the amount of iron in the rats' kidneys. This is consistent with a study by Yatmark et al. (2016) that found that giving rats iron dextran can lead to an accumulation of iron in their kidney organs' tubules and glomeruli. Conversely, administering deferoxamine and deferiprone, two synthetic iron chelators can dramatically lower iron levels.

Outstanding results were obtained in this investigation when sappan wood extract was used as an adjuvant iron chelator. Using the iron overload model, sappan wood extract was able to lower the amount of iron in the rats' kidneys in the group that received it as an adjuvant along with deferiprone at doses of 100, 150, and 200 mg/kg bw. These findings suggest that sappan wood extract has adjuvant potential in enhancing the efficacy of deferiprone as an iron chelator, particularly in lowering the risk of deferiprone side effects in the long run and assisting in the reduction of iron accumulation in the kidney organs.

3.3 Kidney Function Levels

The average results of blood serum urea and Creatinin level examination in rats can be seen in **Figure 2.** Based on the one-way ANOVA analysis, there is a significant difference between the treatment groups (Sig. < 0.05).

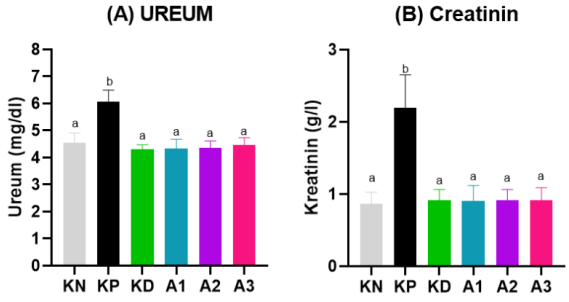

Figure 2. Level of Ureum level (A) and Creatinin (B) in the kidney (KN) Normal Group; (KP) ID group; (KD) DFP + ID ; (A1) SWE 100 mg/kgBW; (A2) SWE 150 mg/kgBW; (A3) SWE 200 mg/kgBW Different letters (a, b, c) indicate significant differences among treatment groups based on the Duncan test ($p < 0.05$).

Urea is one of the indicators used to assess kidney function. Elevated urea levels can indicate glomerular injury (Nabavi et al., 2015). One of the compounds that can lower

urea and prevent kidney damage is antioxidants (Kusmardi et al., 2021). The results of this study show that the group treated with sappan wood extract has lower urea levels. This may be due to the presence of antioxidant compounds in sappan wood extract. Phenolic compounds such as dibenzodioxins, flavones, homoisoflavonoids, chalcones, xanthones, and brazilin found in sappan wood have been shown to have antioxidant potential (Nirmal & Panichayupakaranant, 2015). Based on these findings, sappan wood extract, which contains antioxidant compounds such as dibenzodioxins, flavones, homoisoflavonoids, chalcones, xanthones, and brazilin, has been proven to reduce urea levels and prevent kidney damage.

Creatinine is one of the compounds that can cause toxicity in the kidneys (Kusmardi et al., 2021). In this study, the creatinine levels in the group treated with Sappan wood extract were lower. This result indicates lower kidney damage and dysfunction compared to the other groups. However, the creatinine levels did not differ significantly from the group that was only given deferiprone. This result is consistent with the study conducted by Righi et al. (2021), where the creatinine levels in the group given Salvia verbenaca extract did not differ significantly from the control group that received no treatment. Creatinine levels depend on various factors, including age, muscle mass, and dietary patterns. Therefore, in this case, the decrease in creatinine levels may not be directly related to kidney dysfunction because low creatinine levels can be a sign of decreased muscle catabolism (Delanaye et al., 2017).

Based on the results of the One Way ANOVA statistical test and the Duncan test overall on the measurement of each parameter, the results show that all doses of Sappan wood extract as an adjuvant did not differ significantly from the normal control group and the control group that was only given deferiprone. This indicates that the administration of Sappan wood extract as an adjuvant along with deferiprone is equally effective as giving deferiprone alone. However, in the group where Sappan wood extract was used as an adjuvant with doses of 50 and 100 mg/kg bb, it was more effective in reducing the average percentage of cells undergoing hydropic degeneration, while in the dose of 50 mg/kg bb, it was more effective in reducing the iron content in the kidney compared to the control group that was only given deferiprone. Therefore, it can be concluded that the administration of Sappan wood extract as an adjuvant along with deferiprone can neutralize free radicals caused by excessive iron conditions because one of the causes of hydropic degeneration of cells is the toxicity of free radicals.

3.4 Histopathological Structure

The morphological structure of the kidney organs in the control group and those treated with Sappan wood extract is depicted in Figure 1. The kidneys' histological analysis revealed that the central venta, sinusoids, and hepatocytes' cell structures were all the same in the control group (Figure 5. A). Damage to the hepatocyte cells in the KP group (Figure 5. B) resulted in alterations in cell shape, fatty degeneration, and necrosis in nearly every region that makes up the kidney organ. Though not as much as in the KP treatment group, the KD group's hepatocyte cells nevertheless exhibit changes in parenchymal degeneration, hydropic degeneration, and necrosis with pyknotic nuclei (Figure 5. C). However, the histopathological examination results for groups A1, A2, and A3 (Figures 5.D, 5. E, and 5. F) revealed normal histopathology, even though some cells were still discovered to have necrosis toward the pyknotic nucleus. kidney cells showed no signs of necrosis, the cells in the kidney tubules had normal nuclei (big, round, visible chromatin granules) and full cytoplasm without any large vacuoles. Inflammatory cells that damage organ cells.

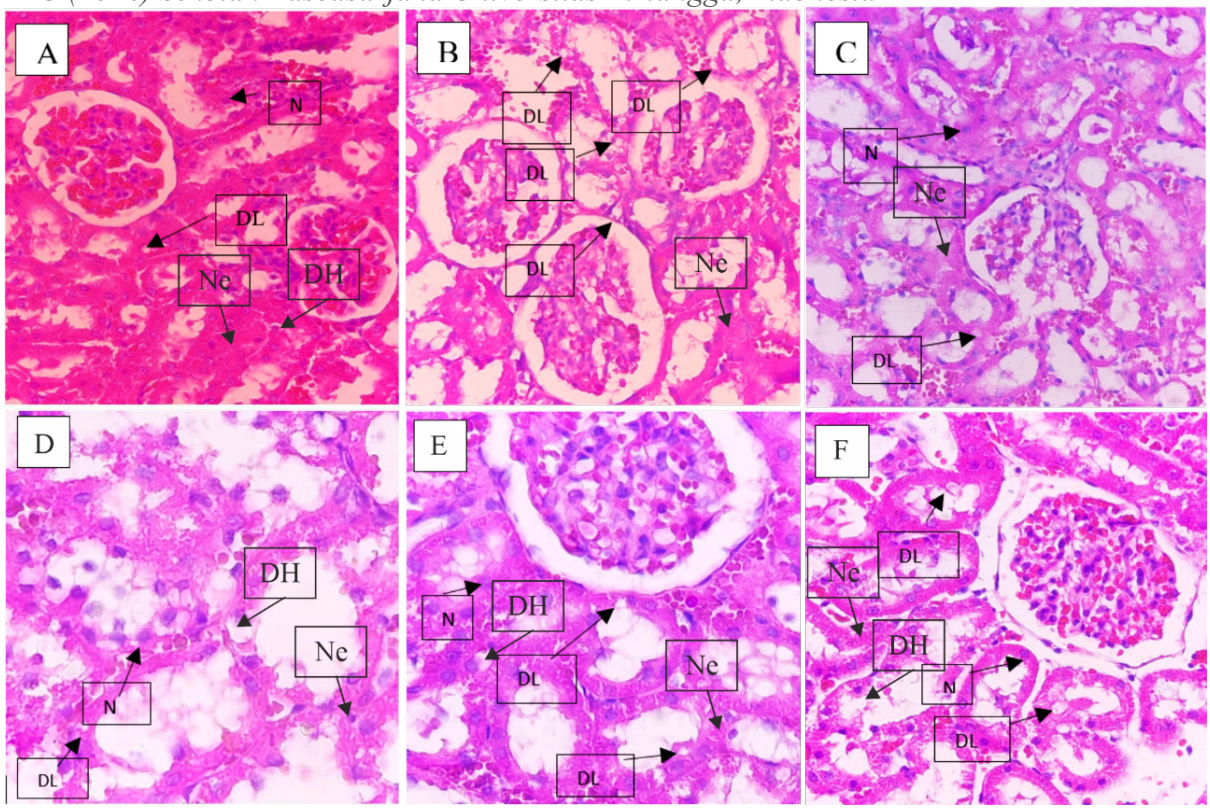

Figure 3. Photograph of kidney histopathology with H&E stain at Tubulus P; 400x]; (A) Normal Group; (B) KP; (C) KD; (E) SWE 100 mg/kg BW; (F) SWE 150 mg/kg BW; (G) SWE 200 mg/kg BW; N = Normal; NE = Necrosis; DL = Lipid degeneration; DH = Hydropic degeneration

Table 1. Histopathological score

Groups	Mean ± SD	P value
KN	158.4 ± 14.67^{a}	
KP	261.4 ± 10.26^{b}	
KD	174.2 ± 8.22^{d}	0.001
A1	178 ± 2^{b}	
A2	184.2 ± 2.94^{bc}	
A3	194.2 ± 3.42^{c}	

Note: Data are presented in mean ± SD, analyzed using ANOVA at the 95% confidence level. Different letters between columns indicate significant differences based on Duncan's follow-up test. Scoring is done by multiplying the number of cells by the damage category. Based on these criteria, the minimum score is 100 if in a normal condition and the maximum possible score is 400 for a necrotic cell condition.

The observations of kidney histological scores in the groups treated with Sappan wood extract and the control group are displayed in Table 1. The group that received iron dextran (KP) had the highest damage score when compared to the other groups, according to the results. Meanwhile, in the KD group and the stiffening group given Sappan wood extract, the kidney damage score decreased to that of the normal control group (KN). Based on the results of the ANOVA test, a p-value of 0.001 was obtained, indicating that there were significant differences in each treatment group. Duncan's follow-up test results showed that the control group had significant differences compared to the other treatment groups. KP was the group with the highest liver damage score compared to other groups.

Necrotic cells are those that alter due to an irreversible process of degeneration, resulting in cell death that can cause harm to surrounding tissue. Cytolysis (loss of cell structure), pyknosis (darkening and shrinking of the cell nucleus), and

keratinization (rupturing of the cell nucleus) are three characteristics of necrosis. Numerous things, including food, infections, the environment, and other elements that enter an organism's body, can lead to necrosis. Serious injuries and the amount of time the organ has been damaged or exposed to toxins, or infectious organisms can all have an impact on how severe the necrosis is. According to Fahrimal et al. (2016), interaction with hazardous substances causes the tubular epithelium to be destroyed, which initiates the necrosis process. Renal tubular epithelial cells can be used for necrosis cell observation. Tubular epithelial cells are extremely vulnerable to harmful compounds entering the kidney since they constantly need a lot of energy for their metabolic processes, according to Lagho et al. (2017).

When iron oxidizes in settings with high iron levels, inflammation may worsen. This illness has the potential to raise blood levels of interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (Ghaith et al., 2022). According to a study by Ige et al. (2019), elevated blood levels of TNF-α and IL-6 as a result of oxidative stress in a model generated by iron overload contribute to the pathophysiology of necrosis in renal tubular cells. In this study, the group receiving Sappan wood extract had a significantly lower necrosis score than the iron dextran-treated negative control group. These findings suggest that Sappan wood extract has the potential to function as an iron chelator, lowering blood iron levels and, consequently, the degree of cell necrosis in rat renal tubules.

4. CONCLUSIONS AND SUGGESTIONS

The administration of Sappan wood extract as an adjuvant is effective in preventing damage to the structures characterized by a decrease in the average percentage of necrotic cells, cells with fat degeneration, and hydropic degeneration, and it prevents the decline in kidney function in rats with iron overload, as indicated by a reduction in urea and creatinine levels. Optimal doses of 50 and 100 mg/kg BW were determined to prevent damage to the structures and kidney function in rats with iron overload.

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