



Molecular Mechanism of Sappan Wood Extract in Rat Liver and Kidney Models with Iron Overload

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Abstract | Iron overload cause chronic transfusion can induce organ damage in liver and kidney. Liver as a main of iron deposite organ and kidney as an iron excretion organ has crucial function in iron metabolism. Reduce negative effect from excess iron in organs before organ damage are needed. The study investigates the impact of sappanwood extract using gene expression analysis in liver and kidney of animal model with *Tfr2*, *Ftl* and *Hamp* as a marker of iron overload. Hepcidin, transferrin, and ferritin genes work synergistically to regulate iron levels in the liver and kidneys. Hepcidin controls the absorption and distribution of iron, transferrin is responsible for the transport of iron to target cells, and ferritin stores safe reserves of iron for use when needed. This regulation is very important to prevent excess iron. We used the rats were housed individually in cages at a temperature and humidity controlled room with a 12-hour light/dark cycle and were allowed free access to deionized water. After an adaptation period of 7 days in our facilities, the rats were randomly divided into 7 groups. We used completely randomized design for seven groups of treatment, SP1 (50 mg/KgBW), SP2 (100 mg/KgBW), SP3 (150 mg/KgBW) and SP4 (200 mg/KgBW) and N as control, ID (iron dextran 60 mg/KgBW) and DFP (Deferiprone 1,8 mg/KgBW). qPCR was used to evaluate relative gene expression of *Tfr2*, *Ftl* and *Hamp* and we used *Gapdh* as the house keeping gene. qPCR profile consist of denaturation at 95°C for 15 seconds, annealing at 58°C for 20 seconds and extention at 72°C for 20 seconds. To counter variability in gene expression, we used house keeping gene such as *Gapdh* and control group in this research. The result indicated that sappanwood extract significantly decreased 1,59 fold of *Ftl* and 1,2 fold of *Hamp* gene expression in the liver and 1,8 fold of *Ftl* and 1,7 fold of *Hamp* gene expression in the kidney at dosage 200 mg/KgBW to ID group. These data indicate that sappan wood extract has high potential as chelator to reduce tissue damage of liver and kidney caused by excess of iron.

Keywords | Chelating agent, *Ftl*, *Hamp*, qPCR, Sappan wood, *Tfr2*

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Chronic transfusions contribute to iron excess (Bruzze *et al.*, 2023). High blood iron levels reduce transferrin saturation by decreasing its binding to iron (Safitri *et al.*, 2018). Iron excess activates hepcidin, an iron metabolism regulator (Yiannikourides and Latunde-Dada, 2019). Hepcidin transduces a signal to ferroportin on liver cell membranes, allowing iron to enter. Ferroportin transports extracellular free iron into liver cells, accumulating free iron (Som and Jodie, 2017). Hepcidin production increases when circulating iron levels are high or when inflammation occurs. This is triggered by high transferrin saturation, which indicates excess iron in the body. By inhibiting ferroportin, hepcidin decreases intestinal iron absorption and reduces iron release from reserves in the liver and macrophages (Sangkhae and Nemeth, 2016). This causes a decrease in circulating iron levels when the body already has enough iron.

Hepatocytes store free iron in the form of ferritin protein but in condition excess iron in the liver, iron can be released into the circulation or used by other cells (Nemeth and Ganz, 2022). However, when iron concentrations are very high, normal regulatory mechanisms cannot keep up, resulting in increased levels of reactive free iron. Free iron can participate in the Fenton reaction, in which Fe^{2+} ions interact with hydrogen peroxide (H_2O_2) to produce hydroxyl radicals ($\bullet OH$), one of the most dangerous forms of ROS (Rauf *et al.*, 2023). ROS activation can induce apoptosis and impairment of liver organ function (Wang *et al.*, 2021) because hydroxyl radicals are highly reactive and can damage cellular components, including lipids, proteins, and DNA, causing oxidative stress (Rios *et al.*, 2023).

Liver and kidney damage can be prevented by binding free iron. Sappan wood is a potent iron chelator. Brazilin is the main bioactive ingredient in sappan wood, an Indonesian plant (Safitri *et al.*, 2022; Harnis *et al.*, 2020). Sappan wood at 200 mg/Kg BW reduces serum ferritin, liver iron, and serum iron by 24.9%, 54.08%, and 38.9%, respectively. It also boosts transferrin saturation and levels by 78.22% and 102.27% in rat iron overload (Safitri *et al.*, 2018). In mice with iron excess, Sappan wood extract, wheatgrass, and vitamin E reduce liver morphological abnormalities and histological damage (Safitri *et al.*, 2017).

Liver and kidney damage can be prevented by binding free iron. Sappanwood extract has major active compound, brazilin. Brazilin is a group of orthodihydroxy catechol and an active compound in Sappan wood (*Caesalpinia sappan*, L.). The molecular structure indicates that brazilin is capable to chelate metal. Sappanwood (*Caesalpinia sappan* L.) is already known as an antichelating iron property by its flavonoids and brazilin compounds in iron overload rats

(*Rattus norvegicus* L.) model. In the previous study showed that various dosages of sappan wood extract were tested start at 50 mg/KgBW, 100 mg/KgBW, 150 mg/KgBW and 200 mg/KgBW. Sappanwood extract at dosage 200 mg/KgBW bw can decreased ferritin, iron liver, and iron serum as 24.9 %, 54.08 %, 38.9 % and increased transferrin saturation, transferrin level, and TIBC as 78.22 %, 102.27 % significantly (Safitri *et al.*, 2018). Sappanwood extract at dosage 200 mg/KgBW dosage was evaluated as the most effective iron chelating properties. In the other research showed that Sappanwood extract can reduce the activity of SOD at 73.78%, lower the MDA levels at 47.91% and increased the activity of GPx at 145.41% and catalase activity at 25.89 % (Safitri *et al.*, 2016).

Antioxidant activity reduces tissue damage, especially in the liver, which stores iron, and the kidney, which excretes iron through urine. Previous research on the antioxidative properties of sappanwood were investigated. Decreased levels of MDA is mainly caused by brazilin compound in Sappanwood extract to capture the hydroxyl radical. The hydroxyl radical can initiate lipid peroxidation reaction (Montensen *et al.*, 2023). Sappanwood extract has active flavonoids and phenolic compounds, 4-O-metilsapanol, protosappanin A, protosappanin B, protosappanin E, brazilin, brazilin, caesalpinia, brazilide A, neosapanone, 7,3,4-trihydroxy-3-benzil-2H (Zhao *et al.*, 2020). As already known in advance that flavonoids have a hydroxyl group that can donate electron to free radicals such as superoxide radicals, hydroxyl radicals and peroxy radicals to be more stable. Lipid peroxidation can be prevented by the flavonoids that also can reduce free radicals, which indicates that flavonoids react with peroxy radicals (ROO^*) and proceed to the termination stage of autoxidation reaction (Valgimigli, 2023). Measurement of antioxidant parameters such as MDA, SOD and GPX4 have the potential to be a sign of oxidative stress in cells. However, these parameters are not directly related to iron chelation activity in cells, so direct measurement of the amount of iron in cells is still needed.

Iron metabolism is indicated by genes and proteins such *TfR2*, *Hamp*, and *Ftl*. Gene expression and regulation are tissue specific, and iron levels affect gene expression directly or indirectly. Many studies have examined the expression of transferrin receptor 2 (*TfR2*), hepcidin (*Hamp*), and ferritin (*Ftl*) genes in animal hepatic and kidney tissues. Transferrin receptor 2 (*TfR2*), hepcidin (*Hamp*), and ferritin (*Ftl*) gene expression in the liver and kidney of iron excessive sappanwood extract exposed rat has not been studied. Hepcidin is coded *Hamp*, binds to ferroportin, which is the main transporter for iron export from cells to the circulation (Trivedi and Barve, 2021). When hepcidin binds to ferroportin, ferroportin internalization and degradation occurs, which reduces the release of iron into the blood. Hepcidin synthesis increases when iron levels in the body are high whereas

it decreases in conditions of iron deficiency. High levels of transferrin (*Tfr2*) can stimulate hepcidin synthesis, thus functioning as feedback on iron status (Zhang *et al.*, 2024). Transferrin also helps reduce the amount of free iron that can produce free radicals, thereby protecting organs from the toxic effects of iron. Free iron in the blood can enter to the cell through transferrin receptor and ion Xc^- (Costa *et al.*, 2023). Free iron will be bind by protein ferritin and deposit in the cell. High level of ferritin in the cell are sign of iron overload. So *Tfr2*, *Ftl* and *Hamp* genes were important to be investigated

Wistar rats were used in this study because it has a high genetic similarity to humans, so research results can be more easily interpreted and applied to human conditions. This is important in studies of the effects of excess iron, which can cause various health problems such as liver damage and metabolic disorders. These animals showed a rapid response to experimental treatments, including exposure to high doses of iron. This allows researchers to observe the short-term and long-term effects of iron overload more effectively.

Although sappanwood extract has been studied for its iron chelating abilities, its mechanism is unknown. A thorough understanding of the molecular mechanism is essential for developing drugs as gene therapy targets in the future. The objective of this work is to investigate the impact of sappanwood extract, which acts as a natural iron chelator, on the expression of transferrin receptor 2 (*TfR2*), hepcidin (*Hamp*), and ferritin (*Ftl*) genes. These genes are considered biomarkers of excessive iron stored in the liver and kidney of model animals.

MATERIALS AND METHODS

REAGENTS

This study used *Tfr2*, *Ftl*, and *Hamp* primers from Li *et al.* (2013), synthesized at Macrogen Singapore. Gene expression was studied using Sybrfast No-Rox Lot. No SF582-B116170 qPCR. Sappan wood extract came from PT. Sari Alam Berkah Garut Indonesia.

ANIMALS AND GROUPS

The Rats care and experimental protocols complied with the Animal Research and Inovation Centre Universitas Padjdjaran. Male Wistar rats (120–150 g) were randomly allocated in 7 groups (n=5 animals each). The rats were obtained from PT. BioPharma Indonesia. Rats were housed in cages with wide mesh wire bottoms and were fed a standard laboratory with free access to tap water. Cages were kept in a temperature controlled room ($22 \pm 1^\circ\text{C}$) of 65–70% relative humidity and a 12 h light–dark cycle. Rats were weighed every 7 days to monitor. This study used animals with ethical committee UNPAD 75/UN.KEP/

EC/2023 authorization. Animal intervention refers to the principles of 3R Reduction, Refinement, Replacement and 5F (Five Freedom of Animal Welfare: Freedom from hunger and thirst, Freedom from discomfort, Freedom from pain, injury and disease, Freedom from fear and distress, Freedom to express natural behavior). For 12 days of administration of iron dextran to rats can cause an increase in iron levels in the blood and several organs of the body, but the increase iron in rats do not cause death, so it still suitable to animal welfare. At the time of termination, rats were given anesthesia to reduce pain and help the death process.

EXPERIMENTAL DESIGN

Iron dextran was injected intravenously into rats at 60 mg/Kg BW to overload the liver and kidney. Iron dextran exposure was spaced out by 3 days for the first 12 days to develop excess iron. Intravenous iron dextran induction for 12 days aims to create a condition of chronic iron overload. This method mimics the process of repeated transfusion in humans in cases of thalassemia patients. Sappan wood extract was tested on test animals' excess iron levels in different treatment groups orally. There are seven groups of treatment, N (normal), ID (iron dextran 60 mg/KgBW), DPF (deferiprone 1,8 mg/KgBW), SP1 (Sappan wood extract 50 mg/KgBW), SP2 (Sappan wood extract 100 mg/KgBW), SP3 (Sappan wood extract 150 mg/KgBW), SP4 (Sappan wood extract 200 mg/KgBW). Test animal experiments lasted 28 days and finished on day 29. Rats were fasted for 24 h before being anaesthetized with ketamine and xylazine intramuscularly. Each experimental group's rat's hepatic and kidney organs were removed and placed in a 1.5 mL cryotube. The samples were stored at -20°C for a long time.

RNA EXTRACTION AND qPCR

The R1057T Zymo research Quick RNA Miniprep plus kit isolated RNA. RNA concentration was quantified with the Nanodrop 2000 Thermo Scientific LGM-EIII-06. Meridian Bioscience's SensiFast cDNA Synthesis kit converts RNA into complementary DNA using DNase. Following cDNA template acquisition from each treatment group, primer optimization was performed. Table 1 Primers qPCR (Qin *et al.*, 2013) lists *Tfr2*, *Ftl*, and *Hamp* as target primers for optimization. Meridian Bioscience's SensiFast Sybr No-Rox kit performed qPCR. Two-step qPCR was used to analyze gene expression. Primary validation was performed by optimizing the primer using PCR to obtain the optimal annealing temperature. Primer optimization was performed at an annealing range of 55°C – 60°C . The results of the optimization of the *Tfr2*, *Ftl* and *hamp* primers were shown in a single curve on the melting curve. Primer efficiency was performed by diluting each primer to create a standard curve. Each primer dilution was repeated three times. Normalization of qPCR results was performed using *Gadph* as a housekeeping gene. The Ct values

obtained from the target gene and the housekeeping gene were separated using the ΔC_t formula. The relative gene expression ratio was calculated using the formula $2^{-\Delta\Delta C_t}$ which shows how many times the target gene expression is under experimental conditions compared to the control.

Table 1: Primers qPCR. (Qin et al., 2013)

Genes	Forward 5'-3'	Reverse 3'-5'
<i>Tfr2</i>	Agaggaggaagatagggaggaagg	Accaaccaccaacacagagtcc
<i>Ftl</i>	Gctggcttcttga tgtcc	Cctctc acacct acctctc
<i>Hamp</i>	Ctgctgtctctctgcttctcc	Agttggtgtctctgcttctctcg
<i>Gapdh</i>	Gcacagtcaaggccgagaat	Acctttctca tgggtgtgaa

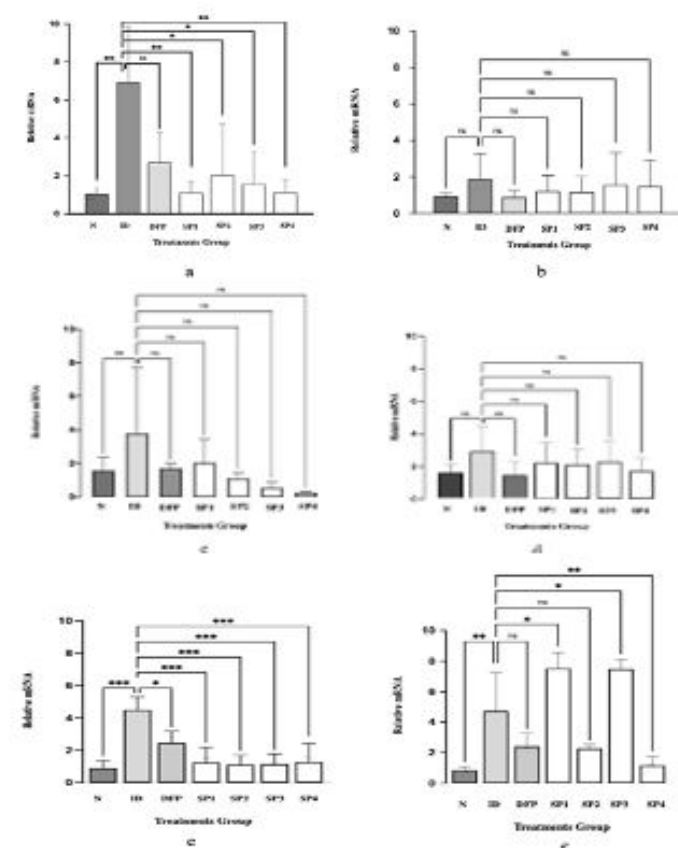


Figure 1: Gene relative expression of *Tfr2*, *Ftl* and *Hamp* in liver and kidney of rats organ in various treatment; Gene relative expression of *Ftl* in liver (a); Gene relative expression of *Tfr2* in liver (b); Gene relative expression of *Tfr2* in kidney (c); Gene relative expression of *Ftl* in kidney (d); Gene relative expression of *Hamp* in kidney (e); Gene relative expression of *Hamp* in liver (f); $P < 0,05$ vs iron dextran.

STATISTICAL ANALYSIS

The RTPCR/qPCR results will be analyzed using Livak and Schmittgen (2001). Data were reported as the mean $\pm$ standard deviation (SD) and were processed using the SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) release 26.0. One-way anova analyses and followed by Pos hoc test for each group comparisons. P-value

< 0.05 was considered statistically significant Data from statistical analysis will be displayed in Graphpad 9.0.

Table 2: Effect sizes and significance levels across different doses.

Organs	Genes	Dosages (mg/KgBW)	Effect size (fold change)	Signicancy level
Liver	Tfr2	50	1	P value < 0.05
		100	2	P value > 0.05
		150	1,8	P value < 0.05
		200	1	P value < 0.05
	Ftl	50	1,2	P value > 0.05
		100	1,2	P value > 0.05
		150	1,6	P value > 0.05
		200	1,6	P value > 0.05
	Hamp	50	1,7	P value < 0.05
		100	1,6	P value < 0.05
		150	1,67	P value < 0.05
		200	1,85	P value < 0.05
Kidney	Tfr2	50	2	P value > 0.05
		100	1	P value > 0.05
		150	0,87	P value > 0.05
		200	0,3	P value > 0.05
	Ftl	50	2,2	P value > 0.05
		100	2,18	P value > 0.05
		150	2,2	P value > 0.05
		200	2,01	P value > 0.05
	Hamp	50	7,4	P value < 0.05
		100	2,1	P value < 0.05
		150	7,4	P value < 0.05
		200	1,3	P value < 0.05

CONSTRUCTION OF PPI NETWORK

GeneMANIA software (<http://genemania.org/>) was used to create a PPI network using an automated weighting mechanism. Different colored edges indicate bioinformatics methods such as co-expression, physical interactions, colocalization, anticipated pathways, common protein domains, and genetic relationships. The GeneMANIA Cytoscape app enables users to construct a weighted composite functional interaction network from a list of genes. Each node represents a gene and its products. The app uses the GeneMANIA algorithm to find other genes and gene products that are most related to the original list, and shows how they are related. The app provides access to most of the features of the GeneMANIA prediction server while removing limitations on gene list length, and the maximum size of the resulting network. The app also allows predictions to be made on user-defined organisms and arbitrarily large custom networks.

Table 3: Regulation of *TFR2*, *FTL* and *HAMP* genes under iron overload conditions in the liver organ.

Proteins Code	Name of Proteins	Mechanisms related to iron-overloaded proteins on the liver	Ref
TFR2	Transferrin Receptor 2	Tfr2 functions as an important receptor in the regulation of iron homeostasis in the liver and contributes to the regulation of hepcidin synthesis, a hormone that controls iron absorption and distribution. When Tfr2 does not function properly, it can lead to excessive iron accumulation and increase the risk of liver damage.	(Pietrangelo, 2016) (Roetto et al., 2018)
FTL	Ferritin Light Chain	FTL can play a role in storing excessive iron and preventing liver parenchymal damage. However, when the cellular storage capacity is exceeded, iron becomes toxic and can cause liver parenchymal damage, as well as increase the risk of liver fibrosis and hepatocellular cancer.	(Piperno et al., 2020)
HAMP	Hepcidin Antimicrobial Peptide	HAMP plays an important role in regulating iron balance in the body. When hepcidin does not function properly, such as in HAMP mutations that cause hereditary hemochromatosis, iron can accumulate in the liver and cause organ damage. One of the HAMP promoters, c.-582 A>G, can affect hepcidin expression and worsen the condition of iron overload in thalassemia major patients.	(Andreani et al., 2009)

RESULTS AND DISCUSSION

Tfr2, *Ftl*, and *Hamp* gene expression was elevated in liver and kidney after iron dextran causing rat iron overload. Deferiprone increased *Tfr2*, *Ftl*, and *Hamp* gene expression in liver and kidney. Sappanwood extract decreased gene expression of *Tfr2*, *Ftl*, and *Hamp* at all dosages compared to iron dextran in liver and kidney animal model (Figure 1 and Table 3).

Postadministration of sappanwood extract, the expression of *Tfr2* and *Ftl* genes in the liver exhibited a notable reduction in comparison to the treatment with iron dextran. These findings indicate that sappanwood extract effectively binds free iron in the liver, therefore decreasing the buildup of iron in the liver, which is the primary organ responsible for iron storage. Nevertheless, the expression of *Tfr2* and *Ftl* genes in the kidneys showed minimal reduction, suggesting that sappanwood extract has not shown effective in decreasing excess of iron in the kidneys, which are responsible for excreting iron. Evidently, the kidneys continue to contain significant amounts of free iron. This disease may result in renal impairment. Conversely, it seems that the *Hamp* gene is significantly upregulated in the liver at dosage 50 mg/KgBW and 150 mg/KgBW. This finding showed that the increase in *Hamp* gene expression in the liver (Figure 1f) at sappanwood extract dosage of 50 mg/KgBW and 150 mg/KgBW indicates that sappanwood extract has not been able to reduce *Hamp* gene expression as expected due to the high iron content in organ. However, at a dosage of 200 mg/KgBW of sappanwood extract decreased *Hamp* gene expression significantly. The decrease of *Hamp* gene expression is highly dependent on the dose of sappanwood extract. The significant decreased in expression of the *Hamp* gene in kidney suggests that hepcidin function effectively as a regulator of iron balance in the kid-

ney than the liver of rats.

Transferrin functions as an endogenous chelator in the body, specifically binding free iron in the blood, while ferritin serves as a storage form of iron in tissues. The *Tfr2* and *Ftl* genes encode these two proteins, which serve as the primary markers of iron chelation in the physiological system (Michael and Stephan, 2019). Elevated levels of free iron in the bloodstream prompt transferrin to effectively bind a significant amount of iron, therefore inhibiting its entry and subsequent buildup in the organs. Therefore, if the concentration of transferrin in the bloodstream is low, the concentration of ferritin in the organs will likewise be low. This research finding showed the potential effect of sappanwood of transferrin and ferritin at the mRNA level and in the future need more exploration using protein analysis method for getting comprehensive result.

Analysis of *Tfr2*, *Ftl* and *Hamp* gene expression at the mRNA level can provide an overview of the activity of transferrin, ferritin and hepcidin proteins in the organ. This can be confirmed by performing histopathological analysis. Perl Prussian Blue staining can color iron in the form of Fe^{3+} and Fe^{2+} in the liver and kidneys in conditions excess of iron. Low expression of *Tfr2* and *Ftl* genes is expected to correlate with reduced iron deposits in the organ as a sign of cell repair due to iron accumulation.

The results of this study demonstrate that a low expression of the *Tfr2* gene in an organ leads to a corresponding decrease in the presence of transferrin. The chemical *Caesalpinia sappan* is classified as a member of the flavonoid class (Nirmal et al., 2015). The expression levels of the *Tfr2* and *Ftl* genes provide evidence of the iron-chelating functionality of *Caesalpinia sappan*. The chelation iron of sappanwood is believed to be derived from the barazilin chemical

Table 4: Regulation of FTR2, FTL and HAMP genes under iron overload conditions in the kidney organ.

Proteins Code	Name of Proteins	Mechanisms related to iron-overloaded proteins on the kidney	Ref
TFR2	Transferrin Receptor 2	TfR2 functions as a sensor for iron status and plays a role in hepcidin regulation. When TfR2 does not function properly, as in the case of hemochromatosis type 3, there is a decrease in hepcidin production leading to iron accumulation in the liver and other organs, including the kidneys. This decrease in hepcidin can contribute to kidney tissue damage due to iron overload.	(Silvestri et al., 2014)
FTL	Ferritin Light Chain	Iron overload causes significant cell death, with an increase in the ferroptosis pathway detected through increased FTL expression. In addition, there was an inverse correlation between the level of FTL protein in urine and renal function, suggesting that FTL may be a useful biomarker for detecting renal damage due to iron toxicity.	(Punchai et al., 2024)
HAMP	Hepcidin Antimicrobial Peptide	HAMP encodes hepcidin, which has a role as a key regulator of iron homeostasis in the body. Hepcidin synthesized in the kidney can affect iron metabolism, especially in conditions of iron overload that occur in kidney disease.	(Pan et al., 2020)

(Safitri *et al.*, 2016), which can bind with iron decreasing the levels of transferrin and ferritin in the liver (Safitri *et al.*, 2017).

Sappan wood extract can reduce the expression of *Tfr2*, *Ftl* and *Hamp* genes as markers of excess iron in rats induced by iron dextran. These are supported by a decreased in transferrin and ferritin levels in the blood and liver of rat model (Safitri *et al.*, 2017). The low expression of the *Tfr2*, *Ftl* and *Hamp* genes is related to the active compound brazilin in sappan wood extract which can bind free iron. The mechanism of iron chelation of sappan wood extract is related to the mechanism of deferiprone. In this research deferiprone reduced gene expression of *Tfr2*, *Ftl* and *Hamp* significantly. Based on that findings, showed that sappan wood extract has similarity mechanism with deferiprone. Another research showed that deferiprone is able to reduce iron levels in the body by increasing the capacity of iron binding protein and reducing NonTransferrin Binding iron (NTBI). Although this study did not measure these two parameters, previous studies have proven it, so sappan wood extract possessed that mechanism.

MECHANISM IRON CHELATION IN LIVER AND KIDNEY

Iron is sequestered in tissues together with ferritin when it is not utilised in cellular metabolic processes. This storage mechanism functions to inhibit the production of harmful redox reactive species by Fe^{3+} ions. In macrophages and liver hepatic cells, ferritin bound iron serves as the primary method for storing iron. Additional cell types, such as erythroblasts, can utilise ferritin-bound iron to facilitate their biological differentiation. Quantifying serum ferritin levels (mg/L) is the most commonly used diagnostic method for evaluating the reserves of iron in the body. Serum ferritin levels typically fall within the range of 20 to 230 mg/L, with variations based on sex and age. Nevertheless, the use of serum ferritin as an indicator of iron store is subject to various constraints (Wang *et al.*, 2021). Additional factors that may influence serum ferritin levels beyond iron

overload include inflammation or tissue damage, which can cause an elevation in serum ferritin levels. Conversely, a deficiency of ascorbate, which can result from iron overload due to fast oxidation of vitamin C, can decrease serum ferritin levels.

The liver is responsible for the synthesis of the preponderance of iron-metabolizing proteins, which is a critical and essential function in iron homeostasis. Hepcidin production and iron homeostasis are primarily regulated by the liver. Hepcidin is expressed in lower quantities in other organs, including alveolar macrophages, pancreatic cells, and the kidneys, in addition to the aforementioned (Yu *et al.*, 2020). The HAMP gene is responsible for the formation of the hepcidin preproprotein. The 25-amino acid-long peptide hormone hepcidin is produced by the intracellular proteolytic cleavage of this preproprotein by the prohormone convertase furin.

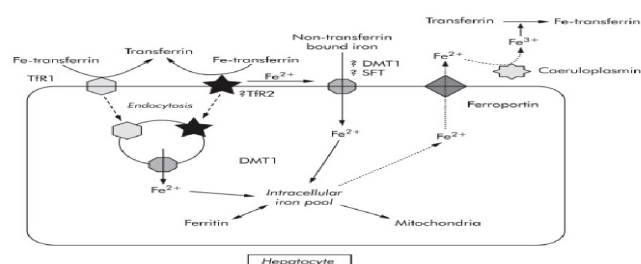


Figure 2: Schematic illustration of the routes by which hepatocytes absorb transferrin bound iron and non-transferrin bound iron (see to the text for further details). Transferrin receptor 1 (TfR1), transferrin receptor 2 (TfR2), divalent metal transporter 1 (DMT1) and iron transport stimulator (SFT). (Trinder *et al.*, 2002).

The liver plays a vital part in the metabolic activities of all cells that respire. The macrophages in the spleen, liver, and bone marrow assume a regulatory and defensive role. By synthesising the hormone hepcidin, which facilitates the recycling of iron from aged red blood cells, it regulates the

renin-epicrin system (RES). Iron is secreted from enterocytes and macrophages into the bloodstream by first passing via the liver (Camaschella and Pagani, 2018). Consequently, this simplifies the process of imposing sanctions and regulatory obligations. Through the synthesis of the hormone hepcidin, it controls the liberation of iron and the preservation of normal plasma iron levels (Muckenthaler *et al.*, 2017).

A number of proteins are hypothesised to function as iron sensors and modulate the production of hepcidin on the hepatocellular membrane. These include the hemojuvelin (HJV), the hemochromatosis protein (HFE), the transferrin receptor 1 (TfR1), and the transferrin receptor 2 (TfR2). The HJV-bone morphogenetic protein (BMP) axis is responsible for the regulation of the production of hepcidin and HAMP (Yiannikourides *et al.*, 2019). BMPRs, which are also referred to as BMP receptors, are located on the membrane of hepatocytes. HJV functions as a co-receptor for BMPs. When BMPs bind to the BMPR-HJV complex, the receptor complex is activated, which in turn stimulates the phosphorylation of cytosolic SMADs 1, 5, and 8. The chemical that accumulates subsequently enters the nucleus, where it interacts with BMP-responsive sites that have been acquired on the HAMP promoter (Van Swelm *et al.*, 2022). Consequently, the HAMP gene is transcribed, resulting in the production of hepcidin. BMP6 is one of the BMPs that are implicated in this system. The non-parenchymal hepatic cells, sinusoidal endothelial cells, hepatic stellate cells, and Kupffer macrophage cells are responsible for its production. When the body's iron levels rise (Jenkitkasemwong, 2016).

PROTEIN INTERACTION TRANSFERRIN, FERRITIN AND HEPESIDIN IN IRON OVERLOAD USING GENEMANIA

The purpose of protein interaction is to determine the interaction between transferrin, ferritin and hepcidin proteins related to iron metabolism. GeneMania is used to predict the interaction of the three genes based on experimental data in the laboratory. The results of the geneMania analysis will show a picture of the results of physical interactions, co-expression, similarities in function and metabolic pathways. This geneMania analysis is to support the results of the in vivo analysis that have been carried out in this study. So that it is expected that the visualization of the results of the in vivo analysis can be described more clearly. Based on PPI network analysis using genemania shows that transferrin, ferritin and hepsidin have co-expression accounts for 8,01%, physical interactions account for 77,64%, colocalization accounts for 3,63%, predicted accounts for 5,37%, pathway accounts for 1,88%, shared protein domains account for 0,60%, and genetic interactions account for 2,87%. The PPI network constructed with GeneMANIA is shown in Figure 3.

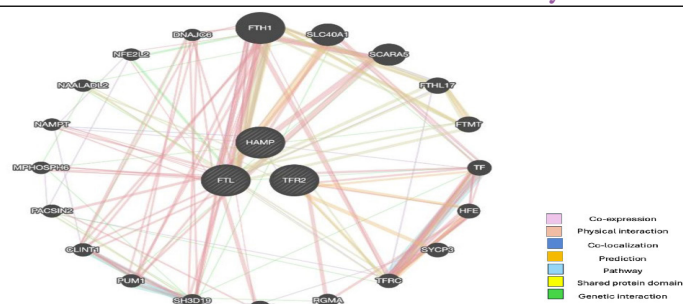


Figure 3: The protein-protein interaction (PPI) network related *Tfr2*, *Ftl* and *Hamp* iron overload constructed with GeneMANIA.

These results showed that *Tfr2*, *Ftl* and *Hamp* genes are in large nodes. This means that the *Tfr2*, *Ftl* and *Hamp* genes interact more often than other genes involved in iron regulation. This supports the results of in vivo research which shows that iron regulation in the liver and kidneys is greatly influenced by *Tfr2*, *Ftl* and *Hamp* genes regardless of the intervention of sappanwood extract.

Co-expression accounts for 8.01%, meaning that the *Tfr2*, *Ftl* and *Hamp* genes have the same expression pattern in conditions of excess iron, influenced by the same transcription factors so that the *Tfr2*, *Ftl* and *Hamp* genes work synergistically in conditions of excess iron. Predicted accounts for 5.37% marked with an orange line means that the *Tfr2*, *Ftl* and *Hamp* genes have the same functional relationship as markers of iron overload. Pathway accounts for 1.88%, meaning that the *Tfr2*, *Ftl* and *Hamp* genes are on the same biological pathway marked with a light blue line. Shared protein domains account for 0.60% marked with a light yellow line means that the *Tfr2*, *Ftl* and *Hamp* genes have the same protein domain that plays a role in DNA binding, enzymatic activity or interaction with other proteins. The *Tfr2*, *Ftl* and *Hamp* genes can bind the same molecule because they have similarities in the protein domain.

Genetic interactions account for 2.87%. This shows the clinical significance related to changes in one gene that affect other genes. The pink line connecting the *Tfr2*, *Ftl* and *Hamp* genes showed that the three genes have a physical interaction of 77.64%. These are involved in signal transduction and regulation of the same gene expression so that they can be used as therapeutic targets in cases of iron overload. Colocalization of 3.63%, which means that the *Tfr2*, *Ftl* and *Hamp* genes at the same location and time. The *Tfr2*, *Ftl* and *Hamp* genes are found in the liver and kidneys in conditions of excess iron.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, we found that sappan wood extract decreased gene relative expression of *Tfr2*, *Ftl* and *Hamp*

as marker of iron overload in liver and kidney of the rats. These results indicated that sappan wood extract has high potential to reduce iron accumulation in liver and kidney. Exploring sappanwood's impact on oxidative stress markers in liver and kidney tissues could provide a comprehensive view. Analysis at the proteins level have opportunity to complete the molecular mechanism of sappanwood extract. In addition, histological observations can strengthen the potential of sappanwood extract in repairing tissue damage due to excess iron.

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NOVELTY STATEMENT

The novelty in this study is the identification of the mechanism of sappanwood extract as a natural iron chelator in the liver and kidneys, identified in a mouse model of excess iron using molecular markers, namely the Tfr2, Hamp and Ftl genes

AUTHOR'S CONTRIBUTIONS

Conceptualization and funding acquisition Ratu Safitri; methodology and writing—review and editing Mas Rizky A.A Syamsunarno; data analysis, original draft preparation, Tanendri Arrizqiyani. All authors have read and agreed to the published version of the manuscript. Racy and Nadhila as a typo editing.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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