



## Iron Chelation Ability of Granule Sappan Wood (*Caesalpinia Sappan*, L.) Extract on Iron-Overloaded

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**Abstract :** The research of chelation ability on two types of granule's formulation of sappan wood (*Caesalpinia sappan*, L) extracts on iron-overloaded rats (*Rattus norvegicus*, L.) has been done. The aims of the study were to determine the effective of formulation and the dose of granulated wooden cup extract to reduce the excess iron in rats indicating the condition of iron-overloaded in thalassemic patient due to blood transfusion. The research has been done using completely randomized design with 11 groups and three replications. Eight groups were administered with two formulations at dose of 0, 100, 200 and 400 mg/kgbw for each formula and the deferipron was taken for control groups. Both groups were given iron dextran. Parameters of iron state measured were the levels of ferritin, and transferrin. Data were analyzed with ANOVA and Duncan Multiple Test Distance. Results showed the administration of iron dextran at a dose of 60 mg/kg bw/day caused excess iron in rats which increased ferritin levels by 70%, and transferrin levels decreased by 33,5% ( $p > 0,05$ ). Granule formula 2 was more effective in reducing excess iron in rats with ferritin reduced by 40% and transferrin increased by 19,1%. Extract dose 200 mg/kg bw /day in the preparation of granules is effective dose in iron chelating with ferritin levels decreased by 30,9% and decreased hepatic iron content of 54,3%. Extract dose of 200 mg / kg bw in the granule preparation is an effective dose for iron chelation with a decrease of 30.9% ferritin levels and transferrin levels 23.74%..

**Key words :** *Caesalpinia sappan* L., granule, iron state, thalassemia.

### Introduction

Thalassemia is a hereditary disease due to imbalance of responsible genes in manufacture of one of the four amino acid chains in hemoglobin synthesis.<sup>[1]</sup> Iron overload is documented in patients with thalassemia. Iron chelator, a molecule forming complex bond with iron ions, is likely the cause to reduce exaggerated levels of iron.<sup>[2]</sup> Iron chelators commonly used are *Desferioxamine*, *Deferiprone* and *Deferasirox*. The third

use of chelator is considered impractical and uncomfortable for patients due to its side effects as well as its high cost.

Phenolic content and flavonoid have iron chelation and antioxidant properties in treating thalassemia patients.<sup>[3]</sup> Dosage of drug used by customer is a very important factor. Form chosen for extract is a health drink cup granulated instant. Granules are clumps of small particles, typically in the form and uneven as single particles with a larger size ranges between 4-12 mesh sieve.<sup>[4]</sup> Granules are easily dissolved, makes it suitable for the preparation of solution.<sup>[5]</sup>

In this study, extract of wooden cup (*C. sappan* L.) was made in instant granules that consists of two formulas. Two types of formula that consists of extract dosage cup with wooden cup (EKS) is different, namely 0, 100, 200 and 400 mg / kg bw. The granules were made by providing enriching substances: fillers, binders and flavoring material, forming a cup extract instant granules. The granules are expected to have activity as iron chelator, to be further used as a herbal medicine for patients with thalassemia.

Measurement on iron chelating activity of instant granules of wooden cup (*C. sappan* L.) was conducted on rats (*Rattus norvegicus* L.) with iron overload. Iron content was determined by measuring ferritin and transferrin levels, and hepatic iron content.

## Materials and Methods

Materials used in the study were distilled water, deferiprone (Ferriprox®), ethanol 96%, Iron Dextran, concentrated HCl, wood dust cup (*Caesalpinia sappan* L), chloroform, mice feed type of CV 151 (laboratory standard), test animals male mice 2-3 months old, weighing between 150-200 grams. The materials used for the manufacture of granules include fumaric acid, citric acid, Avicel PH-102, sodium bicarbonate, Na-CMC, sodium cyclamate and mannitol.

### Preparation of Extract Granules of *C. sappan* L

Skin wooden cup was dried in open air protected from direct sunlight, and grinded to obtain crude drugs sawdust. Wood dust cup was weighed to reach 2.5 kg into a Buchner funnel, macerated using ethanol solvent for 24 hours with three replications. Macerate was further filtered, and concentrated using a rotary evaporator at 40° C forming viscous extract. Raw extract was dried using a freeze dryer to obtain the extract in the form of crystalline powder.

Granular material was made by mixing acid citric acid, fumaric acid, cyclamate, mannitol, Avicel PH 102 and Na-CMC. The mixture was homogenized and dried in an oven (temperature  $\pm$  40 ° C) for 2 days, and continued with the process using print *slugging* tablet with a diameter of 20 mm punch size, after the granulated (mesh '12). The granules were mixed with sodium bicarbonate powder extract and dried in an oven (temperature  $\pm$  40 ° C) until a constant weight.

### Animal Test dan Treatment

Animals used were rats (*Rattus norvegicus*, L.) 2-month-old male with an average body weight of 200 grams. Animals were initially acclimated to environment for one week. During acclimatization, rats were fed with rat feed type CV151 and tap water *ad libitum*.

All treatment of test animals were administered orally, therefore any given substance was made in solution. Granule extracts cup, Iron Dextran and *deferiprone* dissolved distilled water beforehand. Treatments given for 15 days were performed with oral gavage needle. For the provision of Iron Dextran was given in interval of 3 days, whereas less *deferiprone* and granule extract was given daily cup. Treatments given is presented in Table 1.

Table 1 Treatment of Animals Test

Ex	Iron dextran	Formula 1				Formula 2				Deferipron
		d0	d1	d2	d3	d0	d1	d2	d3	
T0	-	-	-	-	-	-	-	-	-	-
T1	+	-	-	-	-	-	-	-	-	-
T2	+	-	-	-	-	-	-	-	-	+
T3	+	+	-	-	-	-	-	-	-	-
T4	+	-	+	-	-	-	-	-	-	-
T5	+	-	-	+	-	-	-	-	-	-
T6	+	-	-	-	+	-	-	-	-	-
T7	+	-	-	-	-	+	-	-	-	-
T8	+	-	-	-	-	-	+	-	-	-
T9	+	-	-	-	-	-	-	+	-	-
T10	+	-	-	-	-	-	-	-	+	-

**Note:**

T0 = rats given distilled water

T1 = The mice were given *iron dextran*

T2 = The mice were given *iron dextran* + deferipron

T3 = Rats were given *iron dextran* + granule formula 1 without EKS

T4 = Rats were given *iron dextran* + granule formula 1 EKS dose of 100 mg / kg bw

T5 = Rats were given *iron dextran* + granule EKS formula 1 dose of 200 mg / kg bw

T6 = Rats were given *iron dextran* + granule EKS formula 1 dose of 400 mg / kg bw

T7 = Rats were given *iron dextran* + granule formula 2 without EKS

T8 = Rats were given *iron dextran* + granule EKS formula 2 doses of 100 mg / kg bw

T9 = Rats were given *iron dextran* + granule EKS formula 2 doses of 200 mg / kg bw

T10 = Rats were given *iron dextran* + granule EKS formula 2 doses of 400 mg / kg bw

**Sample Preparation and Measurement Parameters****Sample Preparation**

Blood samples were carried on day 16 for measurement of transferrin and ferritin, and liver samples were carried for measurement of hepatic iron content (HIC). Blood was taken through a vein in the neck (jugular vein), and collected in 1.5 ml sterile microtube. Blood samples were stored in refrigerator for 10 minutes, and centrifuged at 12000 rpm for 2 minutes. Serum was taken and placed into a new microtube. Serum was stored in the freezer on 20<sup>0</sup> C until use. <sup>[6]</sup>Hearts were aseptically isolated and weighed fresh weight. Film was further inserted into the bottle containing physiological saline.

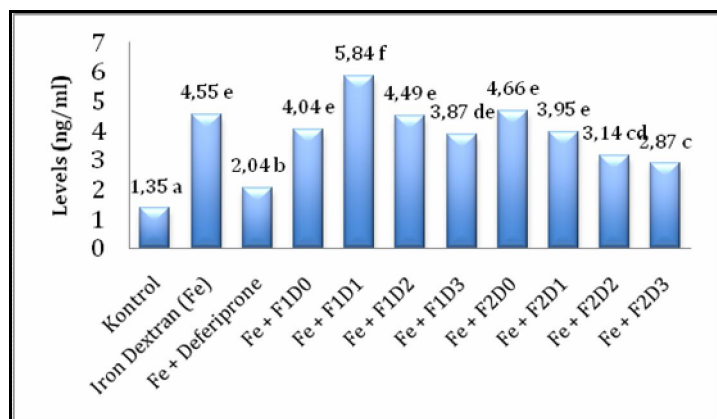
**Measurement of Ferritin and Transferrin**

Ferritin levels were measured using Ferritin Kit (Catalog No. KA 0211), using the ELISA method. Transferrin levels were measured with Transferrin Kit (Catalog No. KA 0510), using the method of liver iron content ELISA. Examination was performed with AAS method (*Atomic Absorption Spectrophotometry*).

**Results and Discussion****Serum Ferritin Levels**

Ferritin assay was conducted to determine the amount of iron stored or its bond to ferritin. Results of analysis of variance (ANOVA) and Duncan's Multiple Range Test showed that there was no significant

difference in ferritin levels each treatment ( $p > 0.05$ ). The average of the results of the calculation are shown in Figure 1.



**Figure 1. Levels of ferritin (ng / dl) Rat (*Rattus norvegicus*, L.) Extract Granules Formula Given Two Secang in Iron Excess Conditions**

Based on the results of Duncan's Multiple Range Test, ferritin levels were lower in control group (1.35 ng / ml) compared to mice with iron dextran (4.55 ng/ml). Ferritin levels were low in mice without treatment in presence of iron content, and iron metabolism in the animal body remains normal reserving ferritin iron in low levels. High ferritin levels were found in mice that received iron dextran, with an increase of 70% compared to controls. High levels of ferritin were obtained in presence of iron dextran in rat's body. Additional iron into the body generates excess iron, while the iron requirement is fulfilled that causes storage in ferritin iron as a backup.

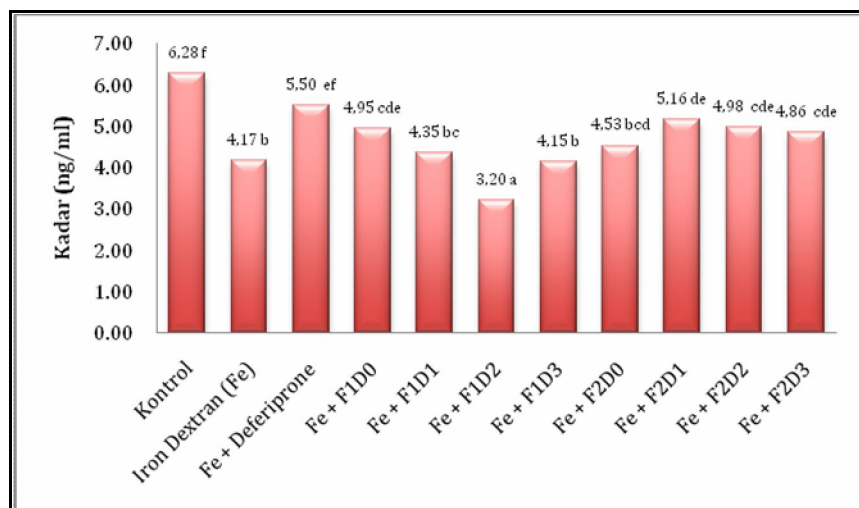
Mice with deferipron exposure showed decreased ferritin of 55.1% compared to iron dextran-given mice. It is associated with the role of deferipron as chelator iron reducing excess iron in the body. In the study, exposure of chelator deferipron caused a decrease in ferritin levels to reach normal values. Deferipron forms a bond with iron atoms and secreted through urine, feces and sweat. Previous study by Galanello (2007)<sup>[7]</sup> showed that administration of deferipron reduced serum ferritin levels in thalassemia patients with excess iron due to repeated blood transfusions.

Exposure of granule EKS showed decreased levels of ferritin along with increasing extract dose, either in formula 1 and formula 2. Higher dose of extract generates ferritin levels approaching normal. Extract granule formula 2 at a dose of 400 mg / kg bw (F<sub>2</sub> D<sub>3</sub>) resulted lower ferritin levels than other formulations, i.e. the percentage decrease of 36.9% compared to the treatment given only iron dextran. It might be caused by flavonoids in the extract granule to play its role chelating metals such as iron. Therefore, to extract a cup, the body of excess iron in the rat as iron dextran can be reduced. Ebrahimzadeh, *et al.* (2008)<sup>[8]</sup> stated that the amount of flavonoids in plant extracts showed a positive correlation to the ability to bind iron.

Results of Duncan's multiple range test showed formula 2 was better than formula 1 in lower ferritin levels, as shown in average of formula 2, ferritin levels were nearly normal than formula 1. These results might be caused by formulation of the respective preparations that affects lower ferritin levels. Analysis of viscosity of tested formula showed formula 2 has a higher viscosity than the viscosity formula 1 indicating the ease of preparations for cast or also in the award of such preparations (for example, when to be taken or injected). Viscosity of the solution will be difficult for the deposition of insoluble particles, so as to disperse the insoluble substances that make it an aqueous suspension. High viscosity is also proposed to increase the stability of the preparation of the solution, involving blocking / troublesome bacteria, fungi or other microorganisms to enter, live and breed in the preparation solution<sup>[9]</sup>

### Transferrin levels

Results of analysis of variance (ANOVA) and Duncan's Multiple Range Test showed that there are different levels of transferrin in each treatment ( $p > 0.05$ ). The mean levels of transferrin any treatment can be seen in Figure 2.



**Figure 2 Transferrin levels (ng / ml) Rat (*Rattus norvegicus*, L) extract granules Provided Secang in Iron Excess Conditions**

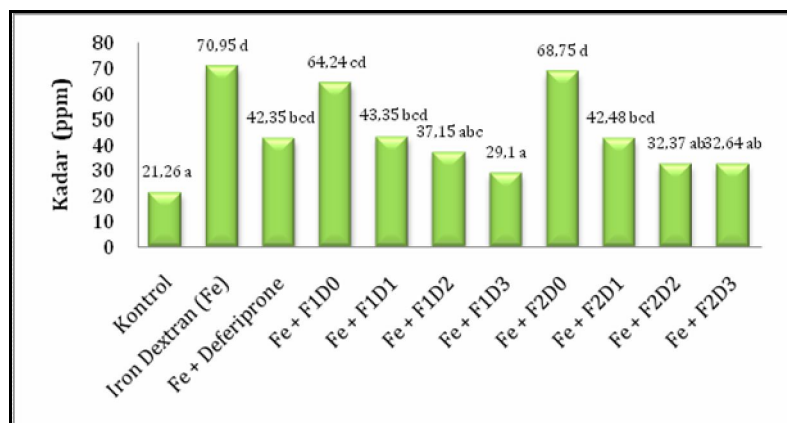
Results of Duncan's Multiple Range Test showed transferrin levels was high in control rats given distilled water (6.28 ng / ml) as well as rats given deferipron. Deferipron is considered very effective in iron chelating. Deferipron increases transferrin levels by 24.1% in treatment of iron dextran. Transferrin was lower than the number of controls is 4.17 ng / ml in rats with administration of iron dextran, and its percentage decreased to 33.5%. These results occur due to excess iron decreasing transferrin level. Previous study done by Benguin *et al.* (1988)<sup>[10]</sup> showed similar finding that an increased transferrin receptor reaches five-fold in iron-deficient mice compared to normal mice, and transferrin receptors in mice with excess iron decreased by 22% compared to normal controls.

The effectiveness of an iron chelator depends on its ability to bind transferrin bound iron is not circulating in the plasma.<sup>[11]</sup> In this study, rats were given chelator deferipron transferrin levels were not different from the control treatment of 5.50 ng / ml. High levels of transferrin indicates that the free iron has been reduced in the plasma. In general, administration of granules formula can increase transferrin level. Administration of granule formula 2 doses of 100, 200 and 400 mg / kg bw showed similar values with deferipron administration, the percentage increase sequentially by 19.1%, 16.2% and 14.1%.

Formula 2 shows the levels of transferrin is more stable than the formula 1. It shows that the granule preparation formula 2 is better than formula 1. Granule formulation also affects its activity in the body, including transferrin level. Granule preparations are considered good if it has good physical properties as well. The formulation can affect the physical properties of the preparation of granules. Measurement of physical properties is important to determine the speed and ease of manufacture, storage and use of a preparation, as well as information for the formulation and formula modification. Recent study by Wijayakusuma (2012)<sup>[9]</sup> showed formula 1 and 2 have different physical properties. Test of physical properties showed effect of formula 2 was faster than soluble formula 1. Lieberman *et al.* (1992)<sup>[12]</sup> states that preparation effervescent requires all components to be easily soluble in water. Short dissolves time indicates a good preparation.

#### **Levels of Iron Liver / Hepatic Iron concentration (HIC)**

Results of statistical analysis of variance (ANOVA) and Duncan's Multiple Range Test showed that there were differences in liver iron levels in each treatment ( $P > 0.05$ ). The mean levels of iron in the liver can be seen in Table 4.3.



**Figure 4.3**The liver iron concentration (ppm) (*Hepatic Iron Concentration*) Rat (*Rattus norvegicus* L) Extract Granules Provided SecangIn Iron Excess Conditions

As shown in figure 4.3, hepatic iron was low in control mice, which was 7.95 ppm. Furthermore, hepatic iron levels in mice treated with iron dextran showed the highest levels, i.e 71.2 ppm. Increased hepatic iron concentration in mice given iron dextran was 70.14% higher compared to controls. High levels of iron in the liver given iron dextran treatment occurs related to liver as the first organ that accommodate excess iron. Therefore, iron in liver damage or liver, contains fairly representative parameters to measure iron status. Referring to study of Ozguner and Sayin (2002)<sup>[13]</sup>, hepatotoxicity is the impact of excess iron generally occurred as liver is main storage of excess iron in the body. In thalassemia, iron accumulation is not only found in the reticuloendothelial cells (Kupffer cells), but also in liver parenchyma (hepatocytes) indicating an increased iron absorption in intestine and repeated blood transfusions.<sup>[14]</sup>

Decreased hepatic iron in mice that received deferipron was 40.3% of the treatment group of iron dextran. Decreased levels of hepatic iron after deferipron treatment might be due to ability of molecule binding to excess iron either in plasma or in tissue. This is consistent with by Ismail, et al. (2010)<sup>[15]</sup> that Deferipron is chelator bidentate iron (three molecules deferipron required to bind one iron atom) which is lipophilic, uncharged, and has a small molecular weight (MW 212). Therefore, in addition of free iron binding in plasma, deferiprone easily penetrate cell membranes in various organs and bind to intracellular iron than other chelator like deferoxamin.

Administration of granule formula and variety of dosage showed hepatic iron concentration measurement results are varied. In general, the formulation does not play a role in decreased iron level in liver that indicates formulation is unable to chelate iron in liver. It can be seen from the hepatic iron content in the given treatment without granule formula 1 and formula 2 without extract granule. Thus, it can be interpreted that decrease in hepatic iron content which allegedly occurred a sailor ability of extract granule contained in the formula.

Overall, a decrease in iron level is found in liver at a given granule treatment with extract granule, both formula 1 and formula 2. Based on the results of Duncan's Multiple Range Test, rats received granule formula on 1 dose of 200 mg / kg bw (F<sub>1</sub> D<sub>2</sub>) and 400 mg / kg bw (F<sub>1</sub> D<sub>3</sub>), formula 2 doses of 200 mg / kg bw (F<sub>2</sub> D<sub>2</sub>), and 400 mg / kg bw hepatic iron levels were not different from control mice.

Ability of granulated extract in reducing liver iron content is associated with chelator compounds contained in extracts granules. Brazilinin extracts cup is a derivative flavonoid which is able to chelate metal ions such as Fe. According to Safitri (2002)<sup>[16]</sup>, brazilin form chelates with Fe<sup>2+</sup> atom in intramolecular and intermolecular. Therefore, iron overload in mice is chelated by chelator compounds in extracts cup.

## Conclusions

Provision of *Iron Dextran* at a dose of 60 mg / kg causes excess iron to ferritin levels increased by 70%, decreased levels of transferrin by 33.5% and increased hepatic iron content of 70.14% compared with the controls. Granule extract preparations wooden cup (*Caesalpinia sappan*, L) formula 2 is more effective in

reducing iron overload in mice with a decrease in ferritin levels by 40% and increase transferrin levels by 19.1%. Extract wooden cup (*Caesalpinia sappan*, L) dose of 200 mg / kg in the preparation of the granules is effective dose mengelat iron with ferritin levels decline of 30.9% and a decrease in hepatic iron content of 54.3%.

### Suggestion

Necessary to study the granule formulation using pure compounds from the extracts have the ability Yag wooden cup mengelat iron.

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