

# THE PROFILE OF SUPEROXIDA DISMUTASE AND MALONDIALDEYDE LEVEL IN THE LIVER TISSUE OF HYPERCHOLESTEROLEMIC RATS TREATED WITH *Holothuria nobilis* POLYSACCHARIDE

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## ABSTRACT

The aim of this research was to analyze the profile of superoxide dismutase (SOD) and malondialdehyde (MDA) on the liver tissue of hypercholesterolemic rats which were given *Holothuria nobilis* polysaccharides (HNP). A total of 15 male rats strain Sprague Dawley were divided into prevention and curative groups. Prevention group consisted of negative/non-hypercholesterolemic group (K-), positive/hypercholesterolemic group (K+), and hypercholesterolemic prevention group which were given 1% cholesterol diet and HNP at dose of 400 mg/kg bw (PCh). The treatments were given for 28 days. The curative group was consisted of the hypercholesterolemic group, which was given 1% cholesterol diet for 28 days, then followed by standard diet for 28 days (Ch), and the hypercholesterolemia curative group which was given 1% cholesterol diet for 28 days, then followed by 400 mg/kg bw HNP for 28 days (ChP). The antioxidant activity of HNP was analyzed by DPPH method. At the end of study the liver tissue was collected and analyzed for MDA, SOD while Cu,Zn-SOD was analyzed by immunohistochemical technique. The results showed that the antioxidant activity of HNP was weak. The MDA level ( $\mu\text{g/g}$ ) in K-, K+, PCh, Ch, and ChP groups were  $1.19\pm 0.6$ ;  $3.37\pm 0.79$ ;  $0.29\pm 0.14$ ;  $9.11\pm 0.72$ ; and  $3.14\pm 1.06$ , respectively. The SOD activities (U/g) in K-, K+, PCh, Ch, and ChP groups were  $2141.11\pm 83.88$ ;  $1541\pm 211.69$ ;  $2096.67\pm 166.66$ ;  $1063.33\pm 88.19$ ;  $1685.55\pm 167.77$ , respectively. The immuno reactivity of Cu,Zn-SOD showed that HNP could increase Cu,Zn-SOD in the liver tissues of both groups. This study concluded that the HNP increased SOD activity, Cu,Zn-SOD antioxidant content, and decreased MDA levels in the liver tissues of hypercholesterolemic rats in both preventive and curative groups.

Key words: hypercholesterolemic, liver, malondialdehyde, polysaccharides, superoxida dismutase

## ABSTRAK

Tujuan penelitian ini untuk menganalisis profil antioksidan superoksida dismutase (SOD) dan malondialdehida (MDA) pada jaringan hati tikus hiperkolesterolemia yang diberi polisakarida *Holothuria nobilis* (HNP). Sebanyak 15 ekor tikus jantan galur Sprague Dawley dibagi dalam kelompok pencegahan dan pengobatan. Kelompok pencegahan terdiri atas kontrol negatif/non-hiperkolesterolemia, kontrol positif/hiperkolesterolemia (K+), dan kelompok pencegahan yang diberi 1% kolesterol dan HNP dengan dosis 400 mg/kg bb (PCh). Perlakuan diberikan selama 28 hari. Kelompok kuratif terdiri dari kelompok kontrol hiperkolesterolemia yang diberi diet kolesterol 1% selama 28 hari, kemudian diikuti oleh diet standar selama 28 hari (Ch), dan kelompok hiperkolesterolemia yang diberi diet kolesterol 1% selama 28 hari, kemudian diikuti oleh 400 mg/kg bb HNP selama 28 hari (ChP). Uji aktivitas antioksidan HNP dianalisis dengan metode DPPH. Pada akhir perlakuan jaringan hati tikus diambil dan dilakukan analisis terhadap kadar MDA serta aktivitas SOD. Kandungan antioksidan Cu,Zn-SOD dianalisis dengan teknik imunohistokimia. Hasil penelitian menunjukkan bahwa aktivitas antioksidan HNP lemah. Kadar MDA ( $\mu\text{g/g}$ ) kelompok K-; K+; PCh; Ch; dan ChP masing-masing adalah  $1,19\pm 0,6$ ;  $3,37\pm 0,79$ ;  $0,29\pm 0,14$ ;  $9,11\pm 0,72$ ; dan  $3,14\pm 1,06$ . Kadar aktivitas SOD (U/g) pada kelompok K-; K+; PCh; Ch; dan ChP adalah  $2141,11\pm 83,88$ ;  $1541\pm 211,69$ ;  $2096,67\pm 166,66$ ;  $1063,33\pm 88,19$ ;  $1685,55\pm 167,77$ . Imunoreaktivitas Cu,Zn-SOD menunjukkan bahwa HNP dapat meningkatkan Cu,Zn-SOD di jaringan hati kedua kelompok. Kesimpulan, HNP meningkatkan aktivitas SOD, kandungan antioksidan Cu,Zn-SOD, dan menurunkan kadar MDA pada jaringan hati tikus hiperkolesterolemia, baik pada kelompok pencegahan dan kuratif.

Kata kunci: hiperkolesterolemia, hati, malondialdehida, polisakarida, superoksida dismutase

## INTRODUCTION

Hypercholesterolemia is a condition characterized by high level of total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C), and very low density lipoprotein cholesterol (VLDL-C) (Elahi *et al.*, 2009). Increased plasma total cholesterol levels are followed by high LDL levels (Hervas and Ascaco, 2018). LDL is a type of lipoprotein easily oxidized. The byproducts of oxidation are in the form of free radicals. Hypercholesterolemia can lead to cardiovascular disease (CVD). Cardiovascular disease (CVD) is a serious problem that commonly occurs in developing countries (Woudberg *et al.*, 2016). Based on data from the World Health Organization (WHO), the prevalence of deaths due to CVD increased to 20 million people in 2015. The Results of Basic Health Research (Riskesdas) in 2013 indicated that the population of Indonesia over the age of 15 years with abnormal cholesterol reached 35.9%. Free

radicals are atoms that have one or more unpaired electrons in their outer orbits. It causes free radicals to be reactive to get their electron pairs. In a certain amount, free radicals are needed by the body to help physiological processes by transferring electrons. However, if there are excessive amounts of free radicals, oxidative stress will occur. The condition of oxidative stress causes physiological and biochemical damages to the body and results metabolic disorders to cell death (Wresdiyati *et al.*, 2006a). Ma *et al.* (2009) reported that the condition of increased oxidative stress continuously decreases the antioxidant system in cardiovascular patients. Hypercholesterolemia is a condition of oxidative stress. Research by Wresdiyati *et al.* (2006a) and Wresdiyati *et al.* (2006b) showed that the condition of hypercholesterolemia in rats causes fat degeneration and decreases antioxidant content of Cu,Zn-SOD in the liver and kidneys. Excessive free radicals in hypercholesterolemia occur as a result of byproducts of

lipid peroxidation reactions and excessive bile formation reactions due to the abundance of cholesterol in the body.

Polysaccharides are biomacromolecules that playing a role on free radical capture, having antioxidant activity and inhibiting lipid peroxidation (Li *et al.*, 2012; Shi, 2016). Liu *et al.* (2002) reported that sea cucumber (*Metriatyla scabra*) has an antihyperlipidemic effect. Polysaccharides from gonad abalone (Zhu *et al.*, 2010), sea cucumber (*Stichopus variegata* Semper) (Yan *et al.*, 2004), and sea cucumber (*Apostichopus japonicas*) (Liu *et al.*, 2012) have also been reported to have antihyperlipidemic effects and antioxidant activity. Other studies reported that polysaccharides have hypolipidemic activities (Tang *et al.*, 2013; Zhang *et al.*, 2013; Xu *et al.*, 2017) by binding to lipids and acting as carriers in cholesterol metabolism to accelerate transport and serum lipid expression (Qi *et al.*, 2017). There has been no report in the antioxidant activity and antihypercholesterolemic of polysaccharides of *Holothuria nobilis*. This study aims to analyze the antioxidant activity of sea cucumber (*H. nobilis*) polysaccharide (HNP), antioxidant content of superoxide dismutase and malondialdehyde level in liver tissue of hypercholesterolemic rats given HNP, both simultaneously with a 1% cholesterol (preventive) diet or given after the condition of hypercholesterolemia (curative).

## MATERIALS AND METHODS

### Preparation of Polysaccharides Sea Cucumber (*H. nobilis*)

Sea cucumber obtained from Ujung Genteng Sukabumi, West Java, Indonesia. Preparation of HNP according to the method Liu *et al.* (2012). Sea cucumber was cleaned and washed, then the meat was taken and cut into pieces. A total of 1000 g of sea cucumber meat was soaked in acetone for 24 hours at 44° C. After 24 hours, the sample was drained and then immersed in 0.1 M sodium acetate buffer (pH 6) for 30 minutes (v/w), containing 5% papain, 5 mL 5 mM EDTA, and 5 mM cysteine, then incubated at temperature of 60° C for 24 hours. The sea cucumber meat was centrifuged (2000 Xg) at temperature 4° C for 15 minutes). Supernatant was taken and added with 5% cetylpyridinium chloride. The mixture was left at room temperature for 24 hours and then was centrifuged (2000 g, 4° C, 15 minutes) to obtain the precipitate. The precipitate was dissolved in a solution of NaCl: ethanol (10/1, v/v) and allowed to stand for 24 hours at 4° C. After 24 hours, the mixture was centrifuged again (2000 g, 4° C, 15 minutes), the supernatant was removed and the precipitate was washed with 95% ethanol. The precipitate was collected and dissolved in 1000 mL of aquadest, filtered, and then evaporated to obtain polysaccharide of sea cucumber.

### Antioxidant Activity Analysis of Polysaccharide Extracted from Sea Cucumber (*H. nobilis*)

The antioxidant activity of *H. nobilis* polysaccharide was analyzed by 1,1 diphenil-2-

pycrylhidrazil (DPPH) method (Sun *et al.*, 2009). The polysaccharides of *H. nobilis* were prepared with different concentrations (1800, 900, 450, 225, and 112.5 ppm) and 2 mL DPPH was added in each concentration. The tubes were incubated in a dark room at room temperature for 30 minutes. The change in absorbance (A) of the sample was measured by a spectrophotometer at wavelength of 517 nm. The antioxidant activity was calculated by the following formula:

$$\% \text{ inhibition} = \frac{(\text{Sample Absorbances})}{(\text{Blank Absorbance})} \times 100$$

Regression equation was obtained from the results of the relationship between the concentration of the sample and the percentage of the inhibition ( $y = bx + c$ ). The equation was then used to obtain inhibitory concentration (IC<sub>50</sub>), which is the amount of sample concentration causing reduction of DPPH compound activity by 50%. The smaller the IC<sub>50</sub> value indicated the higher the antioxidant activity.

### In-Vivo Test of Effect of *H. Nobilis* Polysaccharides in Hypercholesterolemic Rats

In this study, 15 male Sprague Dawley rats aged 2.5 months were used (15±5 g). Rats were obtained from the Drug and Food Inspection Agency (BPOM). The rats were divided into two large groups, namely prevention and curative groups. Prevention group consisted of negative/non-hypercholesterolemic control group (K-), positive/hypercholesterolemic control group (K+), and hypercholesterolemic prevention group which were given 1% cholesterol diet and HNP at dose of 400 mg/kg bw (PCh). Treatment in all prevention groups were given for 28 days. The curative group was consisted of the hypercholesterolemic control group, which was given 1% cholesterol diet for 28 days, then followed by standard diet for 28 days (Ch), and the hypercholesterolemia group which was given 1% cholesterol diet for 28 days, then followed by 400 mg/kg bw HNP for 28 days (ChP). The total treatment in all curative groups was given for 56 days. At the end of the treatment, rats of the prevention and curative group were anesthetized using a combination of ketamine (70 mg/kg bw) and xylazine (10 mg/kg bw). Rat liver was taken to analyze the levels of malondialdehyde (MDA), the activity of superoxidase dismutase (SOD), and the content of antioxidant-Copper, Zinc-Superoxide dismutase (Cu,Zn-SOD).

### MDA Level Analysis of Rats Liver

MDA levels of liver of the rats were analyzed by thiobarbiturate acid reactive substance (TBARS) method described by (Suarsana *et al.*, 2013). The liver was homogenated in saline phosphate buffer (PBS) and then centrifuged (3500 rpm, 20 minutes, at 25° C). Then, the supernatant was taken and was added with 4 mL of cold HCl containing 15% trichloroacetic acid (TCA), 0.38% thiobarbituric acid (TBA), and 0.5% Butylated Hydroxytoluene (BHT). The mixture was then heated at

80° C for 1 hour, then centrifugation (3500 rpm, 15 minutes, at 4° C). Absorbance was measured using a spectrophotometer at a wavelength of 532 nm and a standard solution of 1,1,3,3-tetraethoxypropane (TEP).

### SOD Activity Analysis of Rats Liver

SOD activity analysis according to the method described by Maskar *et al.* (2015). Liver samples were homogenated with phosphate buffer (pH 7) at a ratio of 1:10, then centrifuged (3000 rpm for 10 minutes at 4° C). The supernatant was taken and was added with 0.8 mL of 96% chloroform ethanol (3 : 5), then vortexed and centrifuged (3000 rpm for 10 minutes at 4° C). 100 mL of supernatant were taken and added with 2.8 mL of sodium carbonate buffer pH 10.2 and 100 µL epinephrine solution. Absorbance was measured using a spectrophotometer at a wavelength of 480 nm in the 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> minutes.

### Liver Tissue Processing

Liver Tissue Processing according to the method described by Prasetyawan *et al.* (2017). The liver was washed with 0.9% physiological NaCl and fixed in Bouin's solution for 24 hours, then the liver was put into a 70% alcohol solution as a stopping point. Then, the dehydration process was carried out using alcohol with multilevel concentrations (70%, 80%, 90%, 95% to absolute alcohol I, II, III), then followed by the clearing of the tissue using xylol solution (xylol I, II, III). Furthermore, the tissue was infiltrated with liquid paraffin (paraffin I, II, III), followed by using tissue in paraffin. Then, the tissue block was cut with a thickness of 4 µm using a microtome. The sliced tissue was attached to the glass of the object that had been coated (0.2% neofren® in toluene). Furthermore, immunohistochemical staining of antioxidant Cu,Zn-SOD was carried out.

### Immunohistochemical Staining for Cu,Zn-SOD in The Liver Tissues

Immunohistochemical staining for Cu,Zn-SOD used Cu,Zn-SOD monoclonal antibody (Sigma S2147) (Wresdiyati *et al.*, 2014). The results of the reaction between antigens and antibodies were visualized using diaminobenzidine (DAB) and then counterstained with haematoxylin. The presence of Cu,Zn-SOD was indicated by the appearance of brown color on the nucleus and cytoplasm of hepatocytes. Observations were carried out quantitatively and qualitatively. Qualitative observations were carried out on intensity and distribution the positive reaction of antioxidant Cu,Zn-SOD in the liver tissue. Quantitative observations were carried out on the nucleus of the hepatocytes that giving a positive reaction at various levels of content to Cu,Zn-SOD (strong dark brown or strong positive/+++), medium brown or moderate positive/++ positive, and bluish or weak positive brown/+, and blue or negative/-). The number of nuclei were calculated using the McMaster Biophotonics Image J software program which were then analyzed using analysis of variance (ANOVA).

### Data Analysis

Data in the prevention group were analyzed using one way ANOVA. If the treatment showed differences, then Duncan test was carried out. While data in curative group were analysed using independent T-test.

## RESULTS AND DISCUSSION

### Antioxidant Activity of Polysaccharide Extracted from Sea Cucumber (*H. nobilis*)

The presence of antioxidant compounds in HNP can be determined through analysis of antioxidant activity using 1,1-diphenyl-2-picrylhydrazil (DPPH) method (Prakash *et al.*, 2001). DPPH is a free radical that react with antioxidant by donating hydrogen atoms of antioxidant to this free radicals. The reaction marked by the change of the purple color to pale yellow (Chedea and Pop, 2019). A compound belongs to a weak antioxidant activity if the IC<sub>50</sub> value of the compound is more than 200 ppm (Surinrut *et al.*, 2005). According to Molyneax (2004), the lower IC<sub>50</sub> value showed higher antioxidant activity. According to Ervina *et al.* (2016), a very strong antioxidant activity has IC<sub>50</sub> value less than 50 ppm; strong activity if the IC<sub>50</sub> value between 50 to 100 ppm; medium if the IC<sub>50</sub> value between 101 to 150 ppm, and weak if the IC<sub>50</sub> value is between 151-200 ppm. The IC<sub>50</sub> value of *H. nobilis* cucumber polysaccharide in this study was 1206 ppm. This indicates that *H. nobilis* polysaccharides have a weak antioxidant activity.

### MDA Levels and SOD Activity in Rat Liver Tissue Treatment

The results of the MDA levels and SOD activity analysis of rat liver tissue of prevention group are presented in cytochrome activity. Increased activity of the cytochrome P-450 oxidase will produce excessive free radicals. Cytochrome P-450 acted as a producer of superoxide anion free radicals in endoplasmic reticulum metabolism (Wresdiyati *et al.*, 2008). It was also reported that the condition of hypercholesterolemia increased xanthine oxidoreductase (XO) in producing free radicals (Scheuer *et al.*, 2000). If the production of free radicals occurs excessively, the body antioxidant enzymes cannot handle it. At the end, oxidative stress occurs, in which the amount of free radicals exceeds the amount and antioxidant capacity of the body, so that more free radicals attack lipids, further increasing lipid peroxidation reactions and producing more MDA levels as shown in the hypercholesterolemic group (K+) in this study (Table 1).

The PCh group did not have a significant difference in MDA levels (P>0.05) compared to the negative control group (K-) (Table 1). This showed that the administration of HNP in the PCh group could prevent the occurrence of lipid peroxidation. Inhibition of lipid peroxidation was assumed due to the presence of sulphate polysaccharide in HNP (Wu *et al.*, 2016). Sulfate polysaccharide has various bioactivity; one of them is anticardiovascular (Kiew and Don, 2012). Sulfate polysaccharides from *Ulva pertusa* and *Ulva*

*lactuca* have been reported to have activity as antihyperlipidemia (Sathivel *et al.*, 2008; Qi *et al.*, 2012) which was influenced by molecular weight and sulfate content of polysaccharides (Yu, 2003; Qi *et al.*, 2012). Other studies report that polysaccharides with high sulfate content show stronger antihyperlipidemic activity (Qi *et al.*, 2005; Mestechkina and Shcherbukhin, 2010; Wang *et al.*, 2012).

Sea cucumbers have various types of polysaccharides, including fukoidan and fukosylate chondroitin sulfate. Dong *et al.* (2011) and Zou *et al.* (2016) reported the presence of fukosylate chondroitin sulfate content in sea cucumber (*H. nobilis*). Fukosylate chondroitin sulfate and fukoidan have been reported to be potentially antihyperlipemic (Li *et al.*, 2017) and reduce the risk of atherosclerosis triggered by hypercholesterolemia (Xiu *et al.*, 2018) by a number of mechanisms, including (1) fucosylate chondroitin sulfate which can inhibit HMG-CoA reductase enzyme activity (Riessen *et al.*, 1999); (2) fucosylate chondroitin sulfate which joins with lipids and plays a role in cholesterol metabolism (Wang *et al.*, 2003); (3) fucosylate chondroitin sulfate which acts as a stimulator of bile acid synthesis and encourages lipoprotein lipase (LPL) activity by binding LDL oxidized by polysaccharides (Kaplan and Aviram, 2000). Fukoidan from sea cucumber have been reported to be able to reduce total cholesterol, triglycerides and LDL levels and increase HDL in hyperlipidemic rats (Fitton *et al.*, 2015). Polysaccharide *H. nobilis* is thought to be able to inhibit the activity of the enzyme HMG-CoA reductase, resulting in decreased cholesterol production. The low level of cholesterol in the body causes a low activity of bile synthesis followed by a decrease in the production of free radicals so that the process of lipid peroxidation can be prevented. This caused MDA levels in the PCh group to be significantly lower ( $P<0.05$ ) than that the hypercholesterolemia group (K+); even, the MDA levels were not significantly different ( $P>0.05$ ) from the negative control group (K-).

SOD activity in liver tissue in the hypercholesterolemia group (K+) was significantly lower ( $P<0.05$ ) than that of the negative control group (K-) (Table 1). The condition of oxidative stress in hypercholesterolemia conditions requires more antioxidants to scavenge these free radicals (Zhao *et al.*, 2016). This causes a decrease in SOD activity in rat liver tissue in the hypercholesterolemia group (K+) in this study (Table 1). This was also reported by Zhao *et*

*al.* (2016). Zheng *et al.* (2014) reported a decrease in SOD activity in hypercholesterolemia rats.

The PCh group had the same SOD activity ( $P>0.05$ ) as the negative control group (K-) (Table 1). Inhibition of lipid peroxidation in the PCh group caused SOD levels to be able to neutralize the free radicals that exist in the body. Polysaccharides have an effect similar to Table 1. Table 1 showed that MDA levels in liver tissue of the hypercholesterolemia group (K+) were significantly higher ( $P<0.05$ ) compared to the negative control group (K-). This results showed that the giving a 1% cholesterol diet for 28 days could increase MDA levels. This condition indicated that hypercholesterolemia could cause oxidative stress.

The condition of oxidative stress in hypercholesterolemia occurs through the mechanism of bile acid synthesis. Bile acid synthesis occurred in the liver. The synthesis of bile acids was initiated by reaction of 7-hydroxylase. Catalytic reaction 7-hydroxylase required oxygen, NADPH, and P-450 cytochrome. The condition of hypercholesterolemia increases bile acid synthesis so that more oxygen and NADPH were needed to increase P-450.

Polysaccharides have effect as soluble dietary fiber which is considered a food that cannot be digested. So, polysaccharides can prevent lipid absorption and regulate lipid metabolism in the digestive tract (Gunniss and Gidley, 2010). Other mechanisms, as reported by Li *et al.* (2017), are that fukoidan reduces CD36 activity which functions as a scavenger receptor transferring fatty acids from serum to tissues, reduces the entry of fatty acids into hepatocytes and protects the liver from excessive fatty acids. Excessive fatty acids can encourage peroxisome proliferator-activated receptors (PPAR $\alpha$ ) activity which triggers the oxidation of fatty acids to produce free radicals (Evans *et al.*, 2004). PPAR $\alpha$  increases in response to a high-fat diet and produces reactive oxygen species (ROS) during the fatty acid  $\beta$ -oxidation process that causes oxidative stress (Matsuzawa-Nagata *et al.*, 2008). The administration of polysaccharides in the PCh group is thought to suppress CD36 activity from excessive absorption of fatty acids. Then it will protect the liver from damage caused by fat oxidation. Therefore, giving a 1% cholesterol diet and HNP simultaneously can prevent excessive free radical production so that the PCh group still has high SOD activity (Table 1).

The results of MDA levels and SOD activity analysis of rat liver tissue of curative group were

**Table 1.** MDA levels and SOD activity in prevention group rat liver tissue

Group	MDA ( $\mu\text{g/g}$ )	SOD (U/g)
K-	1.19 $\pm$ 0.60 <sup>a</sup>	2141.11 $\pm$ 83.88 <sup>a</sup>
K+	3.37 $\pm$ 0.79 <sup>b</sup>	1541 $\pm$ 211.69 <sup>b</sup>
PCh	0.29 $\pm$ 0.14 <sup>a</sup>	2096.67 $\pm$ 166.66 <sup>a</sup>

<sup>a, b</sup>Different superscripts within the same column indicate significant differences ( $P<0.05$ )

**Table 2.** MDA levels and SOD activity in curative group rat liver tissue

Group	MDA ( $\mu\text{g/g}$ )	SOD (U/g)
Ch	9.11 $\pm$ 0.72	1063.33 $\pm$ 88.19
ChP	3.14 $\pm$ 1.06*	1685.33 $\pm$ 167.77*

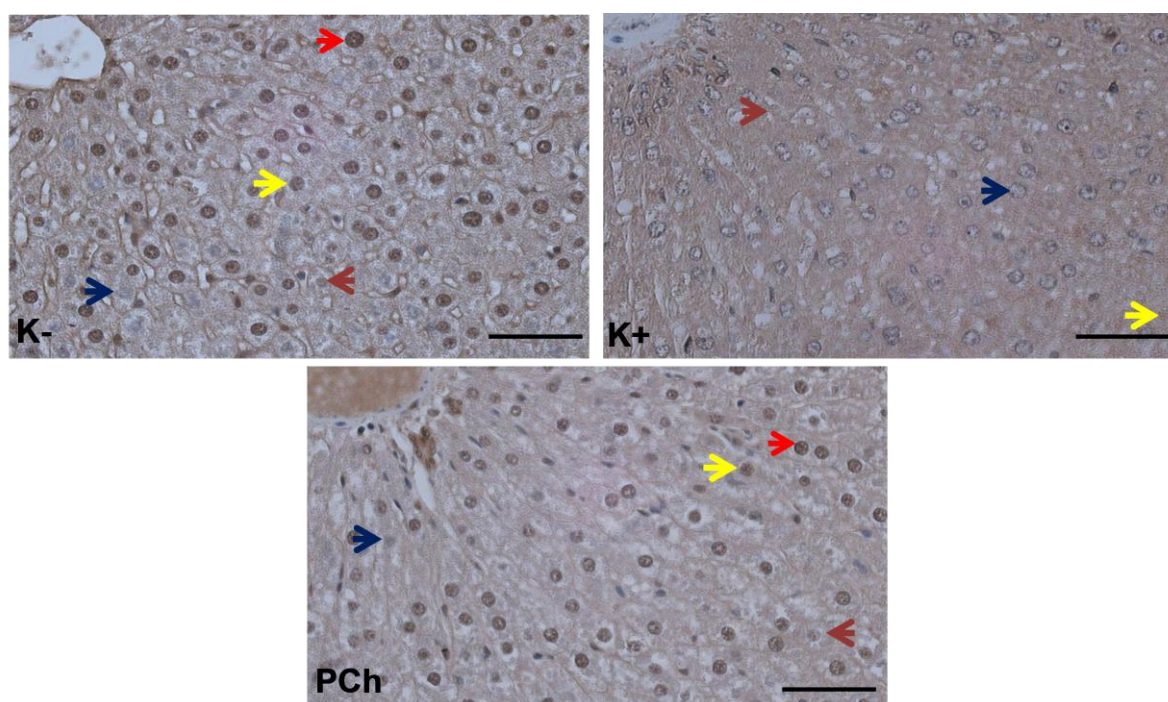
\*Indicate a significant difference ( $P<0.05$ )

presented in Table 2. Table 2 showed that MDA levels of liver tissue in the hypercholesterolemic group followed by standard diet (Ch) were significantly higher ( $P < 0.05$ ) than that of the hypercholesterolemic group followed by the HNP (ChP). Giving HNP in the ChP group could suppress lipid peroxidation so that MDA levels decreased.

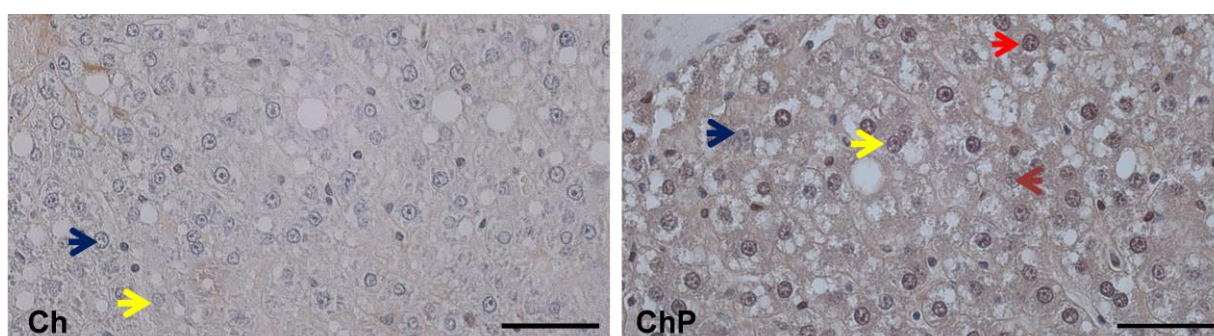
SOD activity in the hypercholesterolemic group (Ch) was significantly higher ( $P < 0.05$ ) compared to that of the HNP treatment group (ChP). The high SOD activity in the ChP group was thought to be due to the fukoidan and fukosylates chondroitin sulfate that have the potential to be antihypercholesterolemic (Dong *et al.*, 2011; Zou *et al.*, 2016). Low SOD activity in group Ch rat liver tissue is a consequence of high free radicals formed from oxidation byproducts in bile formation reactions, so that the body's antioxidants are unable to scavenge these free radicals. This causes a decrease in SOD activity in rat liver tissue in group Ch in this study (Table 2).

### Antioxidant Profile of Cu,Zn-SOD in Rat Liver Tissue

The presence of Cu,Zn-SOD antioxidant showed by brown colour in the liver tissues, using immunohistochemical technique. Qualitative analysis showed that Cu,Zn-SOD content in the liver tissues higher in both preventive and curative rats group (Figure 1 and 2). Quantitative analysis of Cu,Zn-SOD antioxidant content in rat liver tissue was carried out by calculating the number of hepatocyte nuclei at various levels of Cu,Zn-SOD content from immunohistochemical staining (Table 3 and 4). The antioxidant content of Cu,Zn-SOD in the hypercholesterolemic group (K+) was significantly lower ( $P < 0.05$ ) than that of the negative control group (K-) and preventive group (PCh). This is indicated by the number of nuclei which reacts strongly positively (+++) and moderate positive (++) significantly lower ( $P < 0.05$ ), in the K + group compared to that of K- and PCh groups ( $P < 0.05$ ), and the number of hepatocyte



**Figure 1.** Photomicrograph of rats liver tissue in prevention group stained with immunohistochemistry to Cu,Zn-SOD content. K- = Negative control; K + = Hypercholesterolemia; PCh = Given 1% cholesterol + HNP. Red arrow pointed the strong positive, yellow arrow pointed the medium positive, brown arrow pointed the weak positive and blue arrow pointed the negative. 50  $\mu$ m scale



**Figure 2.** Photomicrograph of rat liver tissue in curative group stained with immunohistochemistry to Cu,Zn-SOD content. Ch= Hypercholesterolemia; ChP= Hypercholesterolemia + therapy with HNP. Red arrow pointed the strong positive, yellow arrow pointed the medium positive, brown arrow pointed the weak positive and blue arrow pointed the negative. 50  $\mu$ m scale

**Table 3.** Antioxidant and Cu,Zn-SOD profiles in prevention group rat liver tissue

Group	Number of hepatocyte cells containing Cu,Zn-SOD antioxidant at various level			
	(+++/strong dark brown)	(++/medium brown)	(+/bluish)	(-/ blue)
K-	139.67±10.06 <sup>b</sup>	213±10.53 <sup>b</sup>	163±2.51 <sup>b</sup>	68.67±7.23 <sup>a</sup>
K+	25.67±1.52 <sup>a</sup>	90±6.55 <sup>a</sup>	295.67±6.50 <sup>c</sup>	157±6.02 <sup>b</sup>
PCh	171.11±13.89 <sup>c</sup>	232±18.33 <sup>b</sup>	100.33±7.76 <sup>a</sup>	74±3 <sup>a</sup>

<sup>a, b, c</sup>Different superscripts within the same column indicate significant differences (P<0.05)

**Table 4.** Antioxidant profiles of Cu,Zn-SOD in the rat liver tissue of the curative group

Group	Number of hepatocyte cells containing Cu,Zn-SOD antioxidant at various level			
	(+++/strong dark brown)	(++/medium brown)	(+/bluish)	(-/ blue)
Ch	13.33±2.30	46.50±4.95	126.67±18.23	405±50.79
ChP	102.67±7.50*	275.33±11.93*	145±15.52	105±8.71*

\* Indicate a significant difference (P<0.05)

nuclei that reacted positively weak (+) and negative (-) was higher than that of the K- and PCh groups (P<0.05) (Table 3).

Antioxidant Cu,Zn-SOD is one of the endogenous antioxidants in cells which acts as a defense mechanism by inhibiting oxidation reactions from free radical activity by turning it into a more stable compound (Valko *et al.*, 2006). The content of Cu,Zn-SOD is low in the hypercholesterolemic group (K+) due to the condition of hypercholesterolemia which can produce excessive free radicals. Wresdiyati *et al.* (2006) and Wresdiyati *et al.* (2008) reported that there was a decrease in the Cu,Zn-SOD content in liver tissue of hypercholesterolemic rats.

The high antioxidant content of Cu,Zn-SOD in the preventive group (PCh) was thought to be caused by fukoidan and fucosylates chondroitin sulfate contained in the polysaccharide *H. nobilis*. The administration of fukoidan contained in sea cucumber polysaccharides has been reported to reduce total cholesterol, triglycerides and LDL levels and increase HDL in hyperlipidemic rats (Fitton *et al.*, 2015); it also suppress CD36 expression in the absorption of excess fatty acids to the liver so that fatty acid oxidation can be prevented. This causes the Cu,Zn-SOD in the preventive group (PCh) rats remain high (Table 3).

The quantitative analysis of Cu,Zn-SOD content in the curative group was presented in Table 4. The content of Cu,Zn-SOD in the hypercholesterolemic group followed by HNP (ChP) was significantly higher (P<0.05) compared to that of the hypercholesterolemic group followed by standard diet (Ch). This is indicated by the number of hepatocyte nuclei which reacted strongly positive (+++) and moderate positive (++) in the ChP group was higher compared to that of Ch group. The number of negative (-) hepatocyte nuclei in the ChP group was significantly lower (P<0.05) compared to that of the Ch group. This was indicated by the number of hepatocyte nuclei which reacted negatively (-) in the ChP group was lower compared to that of the Ch group.

The high antioxidant content in the ChP group was assumed due to the presence of fukoidan and fucosylate chondroitin sulfate in *H. nobilis* polysaccharides. Fukosylate chondroitin sulfate and fukoidan have been reported to be potentially antihyperlipemic and reduce the risk of atherosclerosis triggered by

hypercholesterolemia (Xiu *et al.*, 2018). Fukosylate chondroitin sulfate from sea cucumber *Metriatyla scabra* and *Apostichopus japonicas* was reported to have the potential to reduce the risk of atherosclerosis and hyperlipidemia (Liu *et al.*, 2002; Liu *et al.*, 2012). Fukosylate chondroitin sulfate and fukoidan can act as inhibitors of HMG-CoA reductase, accelerate transportation and excretion of serum lipids, stimulate bile acid synthesis, and encourage lipoprotein lipase (LPL) activity by binding LDL oxidized by polysaccharides (Wu *et al.*, 2016). Thus, the antioxidant content of Cu,Zn-SOD in rat liver tissue in the treatment group could be maintained in the hypercholesterolemia group by giving polysaccharide (ChP).

## CONCLUSION

This study concluded that the polysaccharides of sea cucumber (*H. nobilis*) increased superoxidase dismutase (SOD) activity, Cu,Zn-SOD antioxidant content, and decreased malondialdehyde (MDA) levels in the liver tissues of hypercholesterolemic rats in both preventive and curative groups.

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