The Potency Of Pregelatinized Red Bean (Phaseolus Vulgaris L.) Flour On The Improvement Of Lipid Profiles Of Hypercholesterolemic Rats

Waluyo, Yudi Pranoto, Sardjono, Yustinus Marsono

Abstract: The pregelatinization process of red bean (RB) using heating-cooling combination method could improve resistant starch (RS) levels which had the potential to lower cholesterol (hypocholesterolemic). The aim of this study was to determine the effect of pregelatinized RB flour diet on lipid profiles in vivo and to assess the in vitro bile binding capacity. Thirty rats were divided into 5 groups, namely groups of normal rats on standard diet, hypercholesterolemic rats with standard diet (negative control), hypercholesterolemic rats with standard diet were given statin (positive control), hypercholesterolemic rats were on a diet of natural RB flour and hypercholesterolemic rats were on diet of pregelatinized RB flour. Lipid profile analysis was carried out periodically every week during a dietary intervention period of 4 weeks. The bile binding capacity of natural RB flour and pregelatinized RB flour was tested by the method of Soral et al. It was found that pregelatinized RB flour diet reduce total cholesterol levels (-43.66%), LDL cholesterol (-57.80%), triglycerides (-16.32%), and increase HDL cholesterol levels (171.50%). And reduce the AIP (-72.63%). The in vitro study showed that the pregelatinized RB diet was able to bind 13.61% of cholic acid and 48.02% deoxicolic acid. To concloude, pregelatinized RB flour could improve lipid profile and decrease AIP. The possible mechanism was due to the bile acid binding capacity of the pregelatinized RB flour.

Index Terms: Pregelatinization, red bean flour, resistant starch, lipid profile, bile acids, hypercholesterolemic.

1 INTRODUCTION

Dyslipidemia is a major risk factor for coronary heart disease (CHD), stroke and even death, beside other factors [1], [2]. Dyslipidemia is a lipid metabolism disorder characterized by irregularities in the levels of lipid fractions in blood plasma, namely an increase in levels of total cholesterol, LDL and triglycerides, and a decrease in HDL cholesterol [3]. CHD and stroke caused 15.2 million deaths in the world; this disease has become the leading cause of death globally in the last 15 years [4]. Dyslipidemia control can be done by improving the condition of the lipid profile [5], [6]. Cholesterol-lowering drugs such as statin are very dominant in the treatment of dyslipidaemia because more practical [5]. However, the use of drugs can cause adverse side effects, even though it is clinically safe from the medical point of view [7]. Consumption of local food which contains lots of dietary fibre (DF) and RS is an alternative in terms of improving dyslipidaemia. The use of local food has various advantages, including abundant availability so that it is easy to obtain, without adverse side effects and has a wider range of benefits for preventive purposes, promotive and curative. Dyslipidemia conditions, especially hypercholesterolemia can be overcome by consuming foods that contain lots of DF and/or RS [8], [9].

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DF and RS can be obtained from natural plant-based foods, as well as from a variety of refined flour rich in DF and/or RS such as RB flour. RS is a bioactive component that has physiological similarities such as soluble dietary fiber, which is a hypocholesterolemic agent, thereby improving the human lipid profile [10]. The improvement of lipid profile can have an impact on reducing the risk of atherosclerosis which can be seen from the Atherogenic Index of Plasma (AIP) value, which is a way to predict the risk of atherosclerosis [11]. AIP can be calculated with the log formula (TG/HDL). Furthermore, based on the risk of atherosclerosis, AIP is classified into 3 categories, namely low risk (<0.11), moderate (0.11-0.21), and high (>0.21) [12]. One of the mechanisms for reducing cholesterol by dietary fiber and RS was due to its ability to increase bile excretion [8], [13]. The more bile acids bound and excreted with the feces; the less bile acids were recirculated to the liver. It resulted in the synthesis of more bile acids due to the elementary ingredient of bile acids was cholesterol, thus the increased synthesis of bile acids would yield greater reduction in the amount of cholesterol so that it would lower the serum cholesterol level [7], [14]. Pregelatinized RB flour prepared using a combination heating-cooling method (steaming-cooling, microwave-cooling, autoclaving-cooling), contained high RS (12.08%) [15]. Utilization of pregelatinized RB flour to improve lipid profile and its mechanism needed further investigation. This study aimed to identify the potential of pregelatinized RB flour in preventing dyslipidemia through in vivo studies. The effect of RS RB flour on lipid profile was investigated using hypercholesterolemic rats, as induced by a high cholesterol diet. One of the factors that allowed the process of lowering blood cholesterol was studied in vitro by measuring the bile acids binding capacity.

2 MATERIALS AND METHODS

2.1 Materials

The main materials used is pregelatinized RB flour which is

prepared using a combination heating-cooling method (steaming-cooling, microwave-cooling, autoclaving-cooling), with a steaming time of 30 minutes, microwave oven 2450 Hz, 4 minutes and autoclaving 121 ° C, 15 Atm, 145 seconds, and cooling at 4ºC for 24 hours.

2.2 Experimental Animals

The male Sprague Dawley (SD) rats, 2 months old, 150-200 g body weight as many as 30 animals were adapted, fed with standard feed ad libitum for 4 days. Then, the rats were weighed, and their blood was taken to test the lipid profile of the initial treatment. The rats were grouped into two, namely normal (6 animals) fed the standard AIN 93 M diet [16], and hypercholesterolemia (24 animals) were given the hyper cholesterol diet for 7 days. The composition of the hypercholesterolemic feed was the same as the standard feed but contained 10 g/1000 g of cholesterol and 2.5/1000 g of Na cholate by weight of the feed (replacing 12.5 g of corn starch) [17]. To determine the condition of cholesterol had been achieved, a lipid profile analysis was carried out with the criteria for blood cholesterol levels >144.76 mg/dl [18]. Furthermore, the rats were divided into 6 groups, namely the normal group with standard AIN 93 M diet (D1), negative control group namely hypercholesterolemic rats with standard AIN 93 M diet (D2), positive control group namely hypercholesterolemic rats with standard AIN 93 M diet with statin 3 ml / 200 g (D3) [19], natural RB group (D4): hypercholesterolemic rats fed with natural (non pregelatinized) RB flour, pregelatinized RB group (D5): hypercholesterolemic rats fed with pregelatinized RB flour. Feed intervention was carried out for 4 weeks by being given food and drink ad libitum (Table 1). During the intervention, the remaining feed was weighed daily, the rats were weighed, and their blood collected through the eye vessels (retro orbital plexus) for analysis of the serum lipid profile which included triglycerides, LDL cholesterol, HDL cholesterol and total cholesterol once a week. Handling of experimental animals (rats) during the study had approved from the Ethics Commission of the Faculty of Medicine UGM under reference KE / FK / 1221 / EC / 2019.

2.3 Lipid Profile Analysis

During the intervention, blood samples were drawn weekly until the end of the trial (4 weeks). Blood samples were obtained from rats' eyes (retro orbital plexus). Serum was separated from blood cells by centrifugation at 2,000 rpm for 10 minutes and immediately used for lipid profile test using a diagnostic kit. Analysis of total serum cholesterol used the enzymatic colorimetric test method CHOD-PAP [20], with the principle: cholesterol and its esters were released from lipoproteins by hydrolysis reaction by the cholesterol esterase enzyme. The resulting cholesterol was oxidized by cholesterol oxidase to produce H2O2. Furthermore, H2O2 was reacted with 4-amino-antipyrin and phenol by the peroxidase enzyme to produce colored quinonimine. The resulting color was calculated its absorbance. Calculation of total cholesterol levels was done by using the following formula:

Total Cholesterol level (mg/d) =
Sample absorbance /Standard absorbance X Standard concentration (mg/d)

The measurement of HDL serum used the CHOD-PAP method [21]. Precipitation was carried out prior to low density lipoproteins (LDL and VLDL) and chylomicrons. Precipitation was done by the addition of phosphotungstic acid and the presence of magnesium ions (MgCl2). After centrifugation, the HDL in the supernatant was measured using the same reagent kit as the total cholesterol oxidase-p-aminophenozone (CHOD-PAP) cholesterol measurement. The precipitation procedure started with the following steps: as many as 200 □l blood serum mixed with 500 □l precipitation reagent diluted with aquabides (ratio 4 + 1), then incubated for 10 minutes at room temperature. After centrifugation at 4,000 rpm for 10 minutes, a supernatant was produced that was ready for HDL analysis.

HDL level (mg/d) =
Sample absorbance /Standard absorbance X Standard concentration (mg/d)

Triglyceride analysis was performed at initial intervention, 7 days, 14 days, 21 days, and 28 days after intervention, using the Enzymatic colorimetric test GPO-PAP method [22]. The principle of this method was that the triglycerides were enzymatically hydrolyzed to produce glycerol, then glycerol was processed by the enzymatic reaction of glycerokinase to produce glycerol-3-phosphatase. The glycerol-3- phosphatase produced was oxidized by glycerol-3-phosphatase-oxidase to produce H2O2 which was further reacted with aminoantipyrine and 4-cholrophenol by the peroxidase enzyme to produce colored quinonimine. The resulting color was calculated its absorbance.

Triglycerides level (mg/d) =
Sample absorbance /Standard absorbance X Standard concentration (mg/d)

LDL cholesterol levels were obtained through calculations with the formula Rubenfire et al. [23]. LDL cholesterol = Total cholesterol - triglycerides/5 - HDL cholesterol.

TABLE I
STANDARD FEED COMPOSITION AND TREATMENT FEED (G / KG)

Composition	Diet				
	D1	D2	D3	D4	D5
Protein casein	140.00	140.00	140.00	-	-
NRF	-	-	-	624.00	-
PRF	-	-	-	-	585.00
Corn starch	620.70	620.70	620.70	202.39	294.02
Sucrose	100.00	100.00	100.00	100.00	100.00

Soybean oil	40.00	40.00	40.00	30.39	31.28
CMC	50.00	50.00	50.00	-	-
Mineral mix	35.00	35.00	35.00	8.85	12. 30
Vitamin mix	10.00	10.00	10.00	10.00	10.00
L-cystein	1.80	1.80	1.80	1.80	1.80
Choline bitartrate	2.50	2.50	2.50	2.50	2.50
Total Cal	3802.80	3802.80	3802.80	3,992.26	3918.80
Feed weight (g)	1.000,00	1.000,00	1.000,00	979.93	1036.73

Note: D1 = normal group with standard AIN 93 M diet, D2 = negative control group, D3 = positive control group, D4 = natural RB flour group, D5 = pregelatinized RB flour group. NRF = natural red beans flour, PRF = pregelatinzed red beans flour.

2.4 AIP calculation

An Atherogenic Index of Plasma (AIP) was calculated using the method of Niroumand et al. with the formula: AIP = log (TG / HDL) [24].

2.5 AIP In Vitro Bile Acid Binding Capacity

The binding capacity of bile acids (cholic acid, deoxycholic acid) was measured using the method of Soral et al. [25]. The 100 mg sample was mixed with 10 mL of bile acid solution prepared in 0.1 mol of phosphate buffer pH 7.6 for each bile acid at a concentration of 2 µmol/mL. Samples and blanks were incubated at 37 ºC for 30 minutes. Then, centrifugation was performed at a speed of 2,000 g for 5 minutes. A total of 50 µL of sample was mixed with 70% sulfuric acid and 1 mL of fresh furfural solution (2.3 g/L). The absorbance was measured at λ 510 nm after 80 minutes. The results were expressed as %bile acid absorption.

2.4 Statistical Analysis

The study was conducted with 6 treatment factors, namely D1, D2, D3, D4 and D5, with 5 replications. SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis with one-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at a 95% confidence level.

3 RESULTS AND DISCUSSION

3.1 Feed consumption Analysis

The average daily feed intake of the rat group for 28 days of intervention for D1, D2, D3, D4 and D5 were 12.80±1.46 g, 13.49±1.22 g, 13.41±1.40 g, 6.67±1.69 g and 13.66±0.88 g respectively. The natural RB flour diet group (D4) had the lowest level of feed intake compared to the other diet groups.

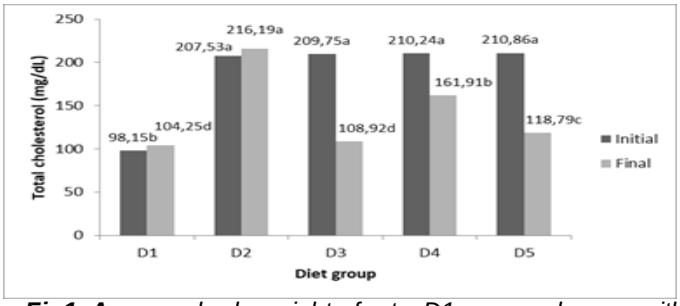

***Fig1. Average** body weight of rats. D1 = normal group with standard AIN 93 M diet, D2 = negative control group, D3 = positive control group, D4 = natural RB flour group, D5 = pregelatinized RB flour group. Different letter notations behind the mean value of the same color indicate a significant difference (p <0.05).*

This difference was thought to be related to the unpleasant smell and taste of natural RB flour. Dimethyl sulfoxide, dimethyl sulfone and ethyl methyl sulfone were responsible for the sulfur odor and causing odor and unpleasant taste in raw kidney beans [26]. The average feed intake for groups D1, D2, D3 and D5 was higher than D4, this indicated that pregelatinized RB flour has an equivalent acceptance rate of standard feed. Processing of pregelatinized RB flour with a heating-cooling combination resulted in high RS content and eliminated the odor and unpleasant taste [15].

3.2 Body Weight.

Changes in body weight among groups of rats during the experiment showed a significant difference ($p < 0.05$) as presented in Figure 1. The D4 diet group had the lowest body weight than the other diet groups. The difference in body weight of the D4 group rats was due to the low level of dietary intake of natural RB flour. Odor and unpleasant taste caused a low level of palatability so that the feed intake was low and resulted in differences in body weight. Changes in body weight of rats in groups D1, D2, D3 and D5 were higher than D4, this indicated that pregelatinized RB flour had a growth impact that was equivalent with standard feed during the treatment.

3.3 Total cholesterol

Total cholesterol levels among the treatment groups showed significant differences ($p < 0.05$) as shown in Figure 2. The total cholesterol levels of D3, D4 and D5 diet groups decreased and it did not occur in the D1 and D2 diet groups. The decrease in total cholesterol on the D5 diet (-43.66%) was higher than the D4 diet group (-23.44%), although it was insignificant to the D3 diet (-48.07%).

These results were like previous studies in that simvastatin had a good effect on lipid metabolism [27]. The decrease in total cholesterol in the D5 diet group was thought to be because of RS levels by 10.52% of total carbohydrates. Another report states that RS consumption of up to 5.4% of total carbohydrates could increase fat oxidation in humans, so that the fat contained in the body will be reduced [28]. Dietary fiber and RS affected in reducing total cholesterol [14]. The mechanism of reducing total cholesterol by RS was thought to be by increasing the excretion of cholesterol and bile acids in feces. The more bile acids bound and excreted with the feces; the less bile acids were recirculated to the liver. This resulted in the synthesis of more bile acids because the elementary ingredient of bile acids was cholesterol. The increased synthesis of bile acids would result in a greater reduction in the amount of cholesterol that would lower the serum cholesterol level [8], [14]. It was also supported by in vitro test

results, the D5 diet had a higher bile acid binding capacity.

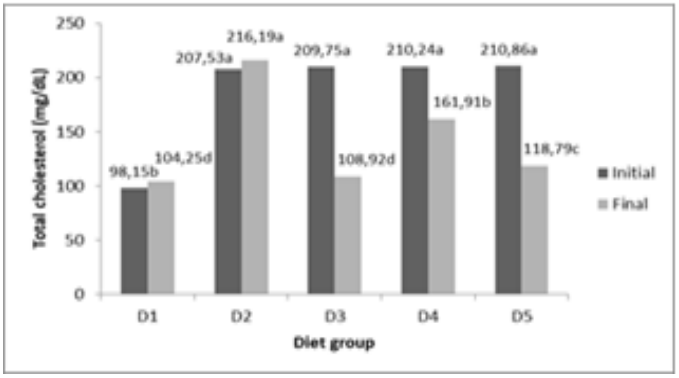

Fig 2. *Average total cholesterol. D1 = normal group with standard AIN 93 M diet, D2 = negative control group, D3 = positive control group, D4 = natural RB flour group, D5 = pregelatinized RB flour group. Different letter notations behind the mean value of the same color indicate a significant difference (p <0.05).*

3.4 LDL Cholesterol

Figure 3 presented the levels of LDL for all groups during the intervention. Levels of LDL in the D1 group the level remained normal, while the D2 group showed an increase (9.86%). Group D5 showed a significant decrease (-57.80%) equivalent to a decrease in group D3 (-57.88%) and group D4 also experienced a decrease (-32.90%). The results showed that pregelatinized RB flour had the same tendency to decrease LDL and total cholesterol. The mechanism for lowering LDL was insignificant to the mechanism for lowering total cholesterol. This reduction in LDL did not differ to the administration of the drug simvastatin. The reduction in LDL cholesterol in the D5 diet group was thought to be due to the high RS levels in pregelatinized RB flour [15]. The ability of RS to bind bile acids affected the contents of the small intestine thicker so that lipid absorption was inhibited and reduced the absorption of bile acids from the small intestine through the enterohepatic circulation. Dietary fiber and RS could increase mRNA receptor of hepatic LDL to increase LDL receptor activity in the liver [29].

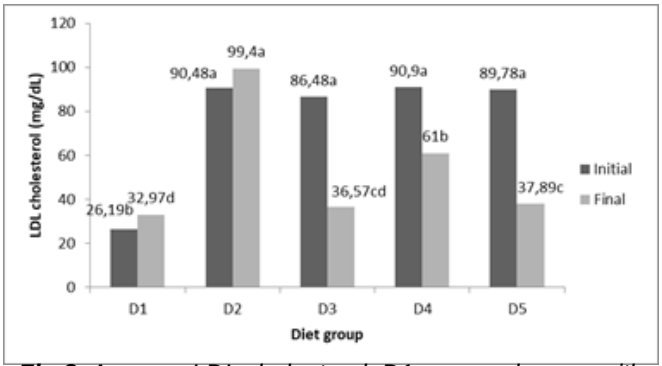

Fig 3. *Average LDL cholesterol. D1 = normal group with standard AIN 93 M diet, D2 = negative control group, D3 = positive control group, D4 = natural RB flour group, D5 = pregelatinized RB flour group. Different letter notations behind the mean value of the same color indicate a significant difference (p <0.05).*

3.5. Triglycerides

Standard Figure 4 showed that the TG levels among the treatment groups showed a significant difference (p <0.05). The TG levels of D3, D4 and D5 decreased and vice versa in the D1 and D2 diet groups increased. The decrease in TG on the D5 diet (-16.92%) was higher than the D4 diet group (-9.80%), although it was significant to the D3 diet (-25.62%). Diets with a higher RS were thought to reduce TG. Soluble dietary fiber could reduce triglyceride levels through inhibition of triglyceride absorption [30]. Other researchers reported that the decrease in triglyceride concentrations was thought to be due to the ability of dietary fiber to increase the rate of bile acid excretion [31]. The decrease in blood TG levels by binding to bile acids by RS was also reported by some researchers [32], [33]. This was confirmed because the ability of RS to had physiological properties similar to dietary fiber [8]. Cholesterol was taken up by the liver and the excess cholesterol for the synthesis of bile and sterol compounds, then cholesterol would be released into the blood circulation in the form of VLDL. In VLDL, there was also TG so that when the VLDL in circulation decreased, the cholesterol and TG in circulation also decreases because they were in one unit of VLDL, and vice versa.

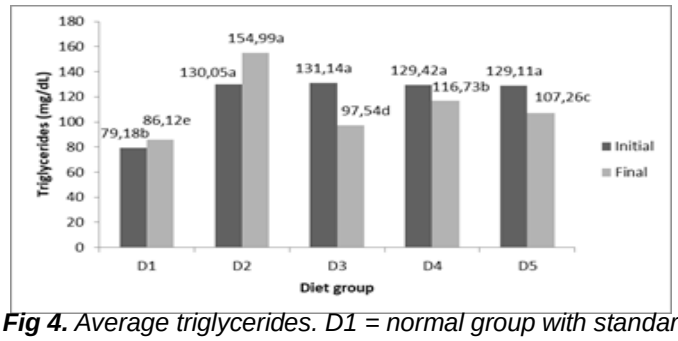

Fig 4. *Average triglycerides. D1 = normal group with standard AIN 93 M diet, D2 = negative control group, D3 = positive control group, D4 = natural RB flour group, D5 = pregelatinized RB flour group. Different letter notations behind the mean value of the same color indicate a significant difference (p <0.05).*

3.6 HDL cholesterol

Figure 5 showed that HDL levels among the treatment groups showed a significant difference (p <0.05). In the D5, D4 and D3 groups there was an increase in HDL, respectively (43.32 mg/dL (171.50%), 36.06 mg/dL (137.58%) and 47.21 mg/dL (186, 82%). In D1 and D2 groups, HDL decreased, namely -5.74 mg / dL (-22.53%) and -0.18 (-0.23%). The increase in HDL in group D5 was higher than in group D4, but still under the D3 group. This was presumably due to the RS content of the D5 diet. The impact of the D5 diet was opposite to the effect on LDL. The results of the intervention during this study explained that HDL increased whereas LDL decreased. In the HDL lipid transport system, it contained a lot of protein and functioned to transport cholesterol from the tissues to the liver so that it could inhibit calcification in the arteries. LDL carried large amounts of cholesterol which could cause calcification in the arteries as a trigger for atherosclerosis [34]. Administration of simvastatin in the D3 group treatment resulted in improved lipid profiles such as increased HDL and decreased total cholesterol, LDL cholesterol and triglycerides [27]. The ability of pregelatinized RB flour to improve lipid profiles such as increasing HDL levels and lowering total cholesterol, LDL and

triglyceride levels was beneficial for heart health. High levels of HDL blood could lower the risk of coronary heart disease [35].

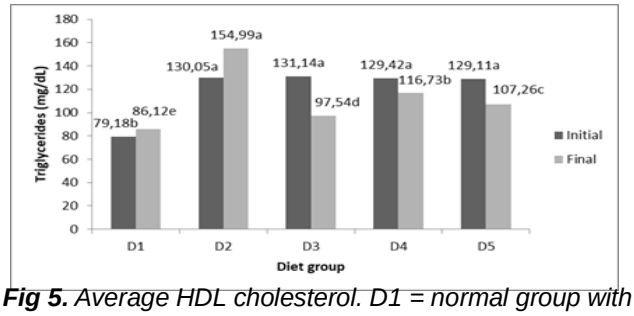

Fig 5. *Average HDL cholesterol. D1 = normal group with standard AIN 93 M diet, D2 = negative control group, D3 = positive control group, D4 = natural RB flour group, D5 = pregelatinized RB flour group. Different letter notations behind the mean value of the same color indicate a significant difference (p <0.05).*

3.7.Atherogenic Index of Plasma

Figure 6 showed the atherogenic index at the end of the intervention period between treatment groups which showed a significant difference (p <0.05). The decrease in AIP occurred in the D5, D4, D3, D1 diet groups, namely -72.63%; -61.09%; -82.19% and -90.24%. In the D2 group, it increased by 25.72%. The decline in AIP in the D5 group was higher than that of the D4 group, but still below the D3 group. This was presumably due to the RS content of the D5 diet. The risk of developing atherosclerosis could be predicted by calculating the AIP [24]. AIP had a significant association with risk factors for cardiovascular disease [36]. According to Dabiasova, AIP could be determined using the log formula (TG/HDL) [12]. The risk of atherosclerosis based on the AIP value could be divided into three groups: low risk (less 0.11), medium risk (0.11-0.21) and high risk (more than 0.21) [37]. At the end of the intervention period, AIP in group D5 was 0. 19 equivalent to group D3 (0.13) and both were still at a moderate risk level for atherosclerosis. AIP in the D4 group of 0.27 also decreased even though at the end of the intervention period it was classified as high risk. This proved the hypothesis that the pregelatinized RB flour diet group could reduce the risk of atherosclerosis by improving the lipid profile

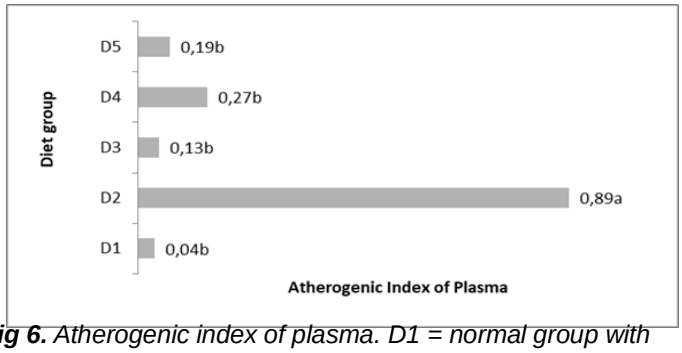

Fig 6. *Atherogenic index of plasma. D1 = normal group with standard AIN 93 M diet, D2 = negative control group, D3 = positive control group, D4 = natural RB flour group, D5 = pregelatinized RB flour group. Different letter notations behind the mean value indicate a significant difference (p <0.05).*

3.8 .Atherogenic Index of Plasma

Figure 7. Showed the bile acid binding capacity among the diet groups which showed a significant difference (p <0.05). This test was carried out in vitro with cholic acid and deoxicolic acid using the cholistyramine comparison standard. The three diets were proven to be able in binding bile acids, both cholic acid and deoxicolic acid. The highest binding capacity of cholic and deoxicolic acids was pregelatinized RB flour diet (D5), followed by the natural RB flour diet (D4) and the standard AIN 93 diet. The three of them are still below the capacity of cholestyramine compounds, namely cholesterol-lowering drugs whose mechanism binds bile acids which are used as a comparison [38]. Cholic acid was a primary bile acid and deoxycholic acid was a secondary bile acid. Deoxycholic bile acids bind more strongly to dietary fiber than cholic bile acids [39].

The ability to bind bile acids from the pregelatinized RB flour diet was thought to come from the soluble fiber and resistant starch. The ability of RB flour to bind to bile acids was affected by the chemical composition and treatment given to pregelatinized flour by a combination of physical methods. This was supported by the results of research which stated that autoclaving treatment was proven to increase the binding ability of dietary fiber to bile acids [40]. Pregelatinized RB flour could bind more bile acids than natural RB flour. This was due to the higher RS content. Similar results reported that the bile acid binding capacity of RS increased with the dose of RS [28].

Soluble DF and RS were viscous so that they could bind bile acids and absorb cholesterol which was then taken to the caecum [14]. The binding and increasing of the fecal secretion of bile acids had been used as a hypothetical mechanism of dietary fiber in reducing cholesterol levels. The more bile acids bound and excreted with the feces; the less bile acids were circulated to the liver. The decrease in bile recirculated to the liver encouraged an increase in the synthesis of bile in the liver, which was the elementary ingredient of cholesterol, so the more blood cholesterol was distributed to the liver, causing a decrease in cholesterol. More bile acid synthesis resulted in a reduction in the amount of cholesterol so that it would lower blood cholesterol levels.

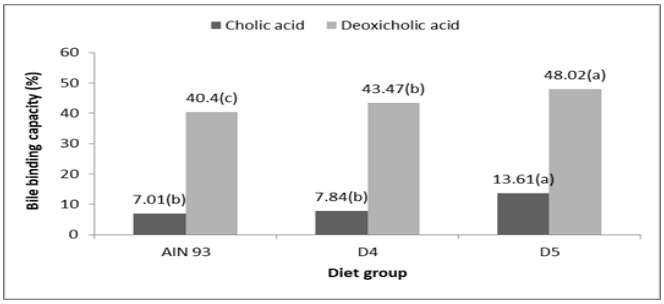

Fig 6. *Atherogenic index of plasma. D1 = normal group with standard AIN 93 M diet, D2 = negative control group, D3 = positive control group, D4 = natural RB flour group, D5 = pregelatinized RB flour group. Different letter notations behind the mean value indicate a significant difference (p <0.05).*

4 CONCLUSION

Pregelatinized RB flour diet (D5) given to hypercholesterolemic Sprague Dawley rats for 28 days resulted a significant improvement in lipid profile (lowering total cholesterol, LDL and TG and increasing HDL). Pregelatinized

RB flour diet was also able to lower AIP. The results of in vitro studies showed the ability of pregelatinized RB flour diet to bind more bile acids. It proved the hypothesis that hypocholesterolemic potential of pregelatinized RB flour through the binding mechanism of bile acid

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