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Decreased total cholesterol levels in rats administered with chitosan from Green mussel (*Perna viridis* L.) shells

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ABSTRACT

Chitosan has been known to have anti-cholesterol activity. This linear polysaccharide can be derived from the chitin of green mussel shells by deacetylation. The purpose of this research was to find out the effects of administering chitosan from green mussel (*Perna viridis* L.) shells on total cholesterol levels. Chitosan was prepared in three steps, namely deproteinization, demineralization, and deacetylation. FTIR was used for characterization, and the absorbance values were calculated to obtain the degree of deacetylation. A total of 24 male Wistar rats were fed with high-fat ingredients (yolk, quail, used cooking oil) and 1% PTU for 30 days p.o and divided into six (6) groups, namely the normal control group, negative control (PGA 1%), positive control (Simvastatin at 0.9 mg/kg BW), Dose 1 (chitosan at 250 mg/kg BW), Dose 2 (chitosan at 500 mg/kg BW), and Dose 3 (chitosan at 750mg/kg BW). The chitosan of green mussel shells had a deacetylation degree of 43.05%. The results showed that the three doses of chitosan exhibited reduced total cholesterol levels in the test rats. At a dose of 750 mg/kg BW, chitosan led to the most significant reduction of total cholesterol levels in rats from averagely 127.1 to 74.2 mg/dL.

Keywords: chitosan, Green mussels (*Perna viridis* L.), rat, total cholesterol

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INTRODUCTION

Hypercholesterolemia is a type of lipid disorder characterized by increased levels of cholesterol in the body. High cholesterol levels cause the incidence of several diseases, such as coronary heart disease (Jannah et al. , 2018). In 2008, the global prevalence of increased total cholesterol in adults was 37% for men and 40% for women (World Health Organitazion, 2018). Statins are the first-line therapy used to reduce cholesterol levels (Stone et al., 2014). Several side effects of drug use on liver and muscle function have been reported, including myopathy and rhabdomyolysis (Harvey, R.A., Pamela, 2014). Given the side effects of anti-cholesterol drugs, the development of natural products as an alternative to anti-cholesterol treatment becomes necessary (Jiang et al., 2015). Several studies have shown that dietary fibers, such as pectin and chitosan, can produce strong hypolipidemic effects (Pan et al., 2016).

Green mussels, a type of popular shellfish in Indonesia, are known for their economic values, nutrients, and delicious taste (Arsyi et al., 2018; Eshmat et al., 2014). Combined with the fact that the edible mussel meat constitutes only 30% of the overall weight, green mussel production generates a substantial volume of waste shells that can pollute the environment (Sinardi et al., 2013; Arsyi et al., 2018). Fortunately, these waste shells contain chitin that, when processed by deacetylation, can produce chitosan (Arsyi et al., 2018). Chitosan has biocompatible, biodegradable, non-toxic, bacteriostatic, and fungistatic properties, so it has extensive applications in the pharmaceutical field (Ambore et al., 2014). It can be absorbed by the body through intestinal epithelial cells, which then regulates lipid metabolisms. It is reported to bind lipids in the gastrointestinal tract and excretes them in feces (Zhang et al., 2012).

MATERIALS AND METHODS

Materials

Green mussel shells were obtained from the seafood market at Tasikmalaya City, and their species (*Perna viridis* L.) was determined by the Department of Biology, Padjajaran University. The reagent for total cholesterol analysis was purchased from Diasys (Holzheim Germany).

Methods

Chitin isolation and chitosan preparation

Deproteinization

The pulverized sample was put into a 500mL beaker, added with 3% NaOH solution with a ratio of 3:1 (mL NaOH/g shell), and stirred with a magnetic stirrer for 1 hour. After that, the solution was heated on a hot plate at 80°C for 30 minutes, filtered, and neutralized to pH 7 by washing it using distilled water. The yield of the deproteinization process was then dried in an oven at 60°C (Poso et al., 2015).

Demineralization

Powdered green mussel shells (*Perna viridis* L.) from the deproteinization results were added with 1.25 N HCl solution with a ratio of 3:1 (mL HCl/g shell). The solution was then heated using a hot plate at 75°C for 1 hour. Afterward, it was filtered and neutralized by washing to obtain a pH 7 solution. The neutral residue was dried in an oven at 60°C (Poso et al., 2015).

Deacetylation of chitin

Chitin was processed by deacetylation using the Knorr method (1982) to produce chitosan. Chitin was added with 50% NaOH solution with a ratio of 1:20, heated at a temperature of 90-100° C, stirred for 60 minutes, and left to reach room temperature. Afterward, it was filtered, and the solid retained was neutralized by washing until pH 7 was achieved. The solid waste was then dried in an oven at 60°C (Edward J. Dompeipen, 2013).

Characterization by FTIR

FTIR characterization was used to analyze functional groups and determine the degree of deacetylation of the chitosan obtained. One mg of chitosan was mixed with KBr (1% w/w), then the mixture was pressed into pellets. Afterward, these KBr pellets were inserted into the trailer, and the infrared absorption spectrum was recorded at the wavenumbers of 4000-400 cm^{-1} (Sinardi et al., 2013).

Analysis of degrees of deacetylation

The degree of deacetylation (% DD) was computed from the infrared spectra of chitin and chitosan using the equation below:

$$DD = [\ 1- \frac{A1655}{A3450} \times \frac{1}{1.33}\ ] \times 100\%$$

Description:

A1655 = Absorbance at a wavenumber of 1655 cm^{-1}

A3450 = Absorbance at a wavenumber of 3450 cm^{-1}

1.33 = Constant, obtained from the ratio of A1655 to A3450 for chitosan with full acetylation

High-fat diet and hypercholesterolemia induction

A total of 24 experimental rats were divided into six groups, namely normal group, negative control group (-), positive control group (+), Dose 1 (chitosan was administered at 250 mg/kg BW), Dose 2 (chitosan at 500 mg/kg BW), and Dose 3 (chitosan at 70 mg/kg BW). The hypercholesterolemia was induced by giving the male Wistar rats in all groups a high-fat diet, consisting of yolk 12 %, quail 20%, waste cooking oil 1%, standard feed 67 %), and 1% Propylthiouracil (PTU) for 30 days p.o. The number of experimental rats used in this study was calculated using the Federer formula (Maryanto, 2013):

Test animals

Apart from receiving a high-fat diet and 1% PTU, all groups of the test rats also received different treatments. The negative control group was given 1% PGA for the first 30 days. Then, from Day 31 to 45, Dose 1, 2, and 3 were given standard feed and chitosan at the doses of 250, 500, and 750 mg/kg BW, respectively. Meanwhile, the positive control group was administered with simvastatin at a dose of 0.9 g/kg BW. Chitosan and simvastatin were delivered orally with a feeding tube. At the end of the treatment, all rats were tested for their total cholesterol levels by the CHOD-PAP (Antipyrine Cholesterol Oxidase-Para Amino) method using a photometer. All research protocols that involved test animals are approved by the research ethics committee of Universitas Padjajaran Bandung (No. 223/ UNG6.KEP/EC/2019).

Total cholesterol analysis

The collected blood samples were centrifuged for 10 min. The serum extract was tested using standard assay kits for in vitro quantitative determination of cholesterol in the serum or plasma on the photometric systems of diasys. Mixed 10 µL Serum with 1000 µL reagent and incubated for 10 minutes at 20-25°C or 5 minutes at 37°C. The absorbance values were read at wavelengths of 500 nm and the Hg 546 nm line within 60 minutes against the reagent blank.

Data Analysis

The total cholesterol levels (in mg/dL) obtained by blood biochemical methods using a photometer and the experimental data were analyzed statistically by One Way ANOVA in the SPSS program. If the data were normally distributed, then a post hoc Least Significant Difference (LSD) test was performed to identify any differences between the test groups.

RESULT AND DISCUSSION

Isolated chitin and chitosan

Deproteinization

Deproteinization is the process of removing proteins found in green mussel shells (i.e., 100 grams of powdered shells) using a 3% NaOH solution. In this process, the protein was released to form Na-proteinate that could dissolve and disappear during washing and filtering (Edward J. Dompeipen, 2013). The reactions occurring in the deproteinization stage are presented in Figure 1:

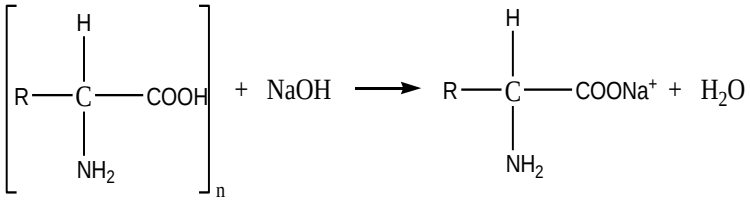

Figure 1. The reaction of deproteinization

The dissolution of protein in NaOH reduced the resultant mass, producing a yield of 90.92 grams.

Demineralization

Green mussel shells contain 33.56% calcium in the form of $CaCO_3$ and $Ca_3(PO_4)_2$. The demineralization stage was intended to remove minerals contained in green mussel shells using strong acids. HCl or H_2SO_4 solutions are generally used for demineralization, but the ability of HCl to dissolve calcium is 10% more effective than H_2SO_4. In this process, the minerals contained in the shells of green mussels reacted with HCl, producing CO_2 gas as an indicator of reaction between HCl and mineral salts contained in the shells (Bahri et al., 2015). The reactions are as follows:

$$CaCO_3(s) + 2HCl(l) \longrightarrow CaCl_2(l) + H_2O(l) + CO_2(g)$$
$$Ca_3(PO_4)_2(s) + 6HCl(l) \longrightarrow 3CaCl_2(l) + 2H_3PO_4(l)$$

The demineralization process produced 75.08 grams of brown chitin powder.

Deacetylation

The deacetylation process was intended to break the acetyl group ($COCH_3$) that was bound to nitrogen in the structure of chitin to enlarge the amine compound ($-NH_2$) in chitosan. This process used a strong base solution, i.e., 50% NaOH. At high concentrations (>40%), NaOH will increase the degree of deacetylation and break the double bond between the carboxyl group and the nitrogen atom (Rochima, 2007). The deacetylation stage used 10 grams of chitin powder and produced 4.00 grams of brownish-white chitosan powder. The yield of the resultant chitosan was 40%, which depends on the % weight of the chitosan against the weight of the chitin derived from green mussel shells.

Characteristics of chitin and chitosan

The absorption bands of the infrared spectra of chitin and chitosan in this research are shown in Figure 2.

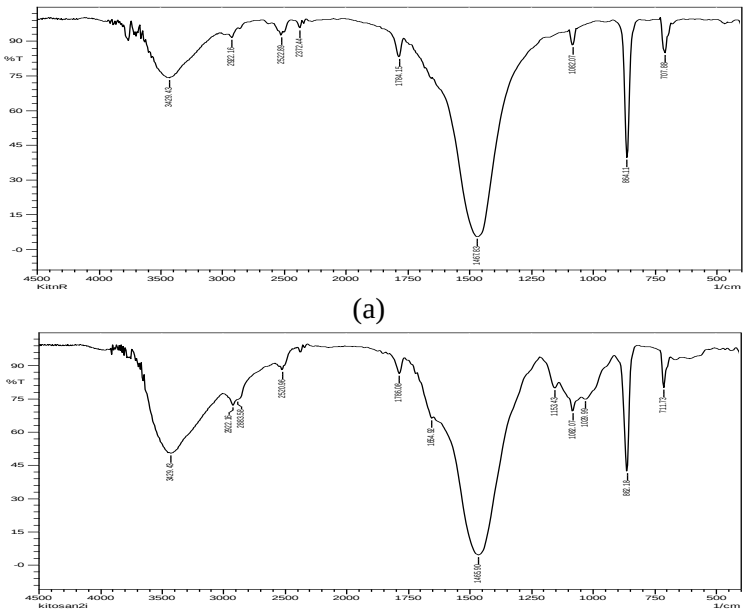

Figure 2. FTIR Spectra of (a) Chitin and (b) Chitosan

FTIR was used to identify the functional groups in chitin and chitosan (Table 1). At a wavenumber of 3429.3 cm^{-1}, the spectra indicated the vibrations of OH and NH groups. The width of the absorption band and the wavenumber shift of the OH group are attributable to the overlapping of the NH group in amines. Moderate absorptions at wavenumbers of 2522.89, 2372.44, and 2520.96 cm^{-1} are thought to be caused by vibration from the primary NH amine strain in chitin. Typical absorption of chitosan was apparent at a wavenumber of 1654.92 cm^{-1}, which shows the N-H bending vibration of amines ($-NH_2$). The vibration of the C=O group at a wavenumber of 1786.08 cm^{-1} proved that the resultant chitosan still contained an acetyl group due to imperfect deacetylation (Silverstein et al., 2005).

Table 1. Identification of the FTIR peaks of chitin and chitosan derived from green mussel shells

Functional groups	Wavenumbers cm-1		References cm^{-1} (Silverstein, 2005)
	Chitin	Chitosan	
OH Stretching NH Stretching	3429.3	3429.3	3650-3200 3500-3400
CH Stretching	2922.16	2922.16	3000-2840
NH Stretching	2522.89	2520.96	2800-2000
NH Stretching	2372.44	-	2800-2000
NH Bending	-	1654.92	1655-1620
C=O Stretching	1784.15	1786.08	1870-1540
CH Bending	1467.83	1465.90	1468-1462
CO Stretching	1082.07	1082.07	1085-1050
C-C Stretching	864.11	862.18	1200-800
CN Stretching	707.88	711.73	800-666

Degrees of deacetylation

The degree of deacetylation of chitosan determines the formation of chitosan from chitin. Ray (2011) states that the degree of deacetylation for chitosan is within the range of 40-98%. The results showed that the degree of deacetylation of green mussel was 43.05% (>40%), meaning that the chitosan obtained in this experiment is usable.

The effect of chitosan on total cholesterol levels

Plasma cholesterols are one of the risks of coronary heart disease and other cardiovascular disorders. The normal total cholesterol levels in humans are <200 mg/dL, while in rats, these numbers range between 46 and 92 mg/dL (Krinke, 2002). In this study, the total cholesterol levels of rats receiving a high-fat diet and 1% PTU increased after 30 days. Then, the total cholesterol levels of rats receiving chitosan for 15 days were measured on Day 45. The results are presented in Table 2.

Table 2. The mean total cholesterol levels of rats in all groups

Groups	Total cholesterol levels after a high-fat diet (mg/dL)	Total cholesterol levels after treatments (mg/dL)	P (<0.05)
N	77.98 ± 9.362	81.2 ± 1.92	
(-)	134.4± 19.155	123.7 ± 10.61	
(+)	128± 8.417	67.2± 1.83	
D1	129.2 ± 12.28	97.55 ± 13.18	0.00
D2	109.7±7.59	75.78 ± 10.15	
D3	127.1 ± 21.51	74.2 ± 1.05	

Notes: (P=0,00): significant deference between groups

Goat fat has 27.14% saturated fat (Susilawati et al., 2015), quail egg yolk contains 2138.17 mg cholesterol per 100 g (Febriani, 2018), and used cooking oil contains 70% saturated fatty acid. Based on the results of the study, high-fat diets made of standard feed mixtures, goat fat, quail egg yolks, and used cooking oil increase total cholesterol levels together with the administration of 1% PTU (propylthiouracil) to induce the excessive presence of cholesterol in the blood. Propylthiouracil is a thyroid hormone antagonist that, when the thyroid hormones are normal, can increase fat metabolism by increasing the formation of LDL receptors in liver cells, resulting in rapid transfer of LDL from plasma and secretion of cholesterol lipoprotein by liver cells. When the thyroid hormone is inhibited, plasma LDL production decreases, and consequently, cholesterol levels increase (Umami et al., 2016).

The administration of chitosan prepared from green mussel shells at a dose of 750 mg/Kg BW rats (Dose 3) showed the lowest total cholesterol levels in all three test groups with an average of 74.2± 1.055 mg/dL, followed by Dose 2 and then Dose 1 (with an average of 75.78±10.151 and 97.55±13.179 mg/dL, respectively). However, when compared to the control group (+), the group receiving Simvastatin (0.9 mg/kg BW), a commercial LDL-lowering drug, showed a higher decrease in total cholesterol levels. The mechanism of simvastatin as anticholesterol involves the competitive inhibition of the enzyme HMG-CoA reductase, which functions as a catalyst in the formation of cholesterol (Umami et al., 2016). The results are presented in detail in Table 2.

Green mussels are a potential extractable source of therapeutic and bioactive compounds (Navalgund and Khan, 2016), including chitosan. Chitosan can reduce cholesterol due to its cationic properties, i.e., the capacity to bind negatively charged lipids, and thereby minimize absorption in the digestive tract and serum cholesterol (Trivedi et al., 2016). The waste shells of green mussels can be utilized for chitosan production because they contain chitin (Danarto and Distantina, 2016). Chitosan can bind fat and remove it with dirt because this linear polysaccharide is a fiber that cannot be digested by the body (Pratiwi, 2014). Chitosan treatment can increase 7α-hydroxylase cholesterol (CYP7A1)

activity in the liver, which is associated with a decrease in levels of plasma cholesterol (Moon et al., 2007). CYP7A1 is a specific enzyme in the liver that catalyzes the biosynthesis of bile acids from cholesterol (Kim and Kim, 2010). When exposed to stomach acid, the -NH_2 group in chitosan will bind to hydrogen ions (H^+) to form a tertiary amino group (NH_3^+), then chitosan forms an ionic complex with fats, fatty acids, other lipids, and bile acids, hampering absorption from the intestine to the liver. Enterohepatic circulatory disorders of colic acid and other bile acids cause an increase in the biosynthesis of cholic acid from cholesterol in the liver, due to which the cholesterol levels of liver cells lower and the LDL-receptor expression increases (Ylitalo et al., 2002). The hypocholesterolemic effect of chitosan tends to increase with degrees of deacetylation (DD). Chitosan with a high degree of deacetylation has more free amino groups that make it more positively charged, so it helps strengthen the binding of fatty acids in the bile duct (Wang et al., 2019).

CONCLUSION

The chitosan derived from the waste shells of green mussels (*Perna viridis* L.) has a degree of deacetylation of 43.05%, and its administration to hypercholesterolemic male Wistar rats has been proven to reduce total cholesterol levels. At the dose of 750 mg/kg BW rats, chitosan gives the most significant reduction of total cholesterol levels compared to 500 and 250 mg/kg BW rats, from averagely 127.1 to 74.2 mg/dL.

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