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The Effect of Squid Bone Chitosan Powder (*Loligo* sp.) on Total Cholesterol Levels of Rats (*Rattus norvegicus*)

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ABSTRACT

Bones in squid (*Loligo* sp.) can be used as a source of medicinal ingredients, one of which is chitosan. The polymer in chitosan contains amino and hydroxyl groups per residue, giving chitosan many biological activities, such as anti-inflammatory, antimicrobial, hypoglycemic, hypocholesterolemic, and immunostimulatory effects. This study aims to determine the antioxidant activity and the effect of chitosan squid bone powder on the total cholesterol levels of rats (*Rattus norvegicus*). This type of research used an experimental method with a one-group pre-test and post-test design, in which 12 male white rats, body weight 150 g, were divided into four groups. Group I was a negative control of 1% Na-CMC, group II chitosan 50 mg/kg, group III chitosan 100 mg/kg, and group IV positive control of simvastatin 10 mg, which each group was given orally; the treatment was carried out for two weeks. The results showed a decrease in the mean after treatment in the treatment group with chitosan powder, but there was no significant difference compared to the control group (P>0.005). This shows that squid bone chitosan powder reduced total cholesterol levels in rats.

Keywords: Chitosan, cholesterol, squid bones (*Loligo* sp.)

Introduction

Indonesia has abundant marine or fishery products. One of the fishery products in Indonesia is squid *(Loligo* sp.*)*. This marine mollusk has been widely used and utilized by the community as a that can be used as a medicinal ingredient. So that the internal squid bones are not used as a cooking ingredient. However, ordinary people still do not know that squid bones contain ingredients just wasted during processing (Rochmawati & Nabila dkk, 2018).

Chitosan is a natural biopolymer with the second-largest abundance after cellulose. It is a product of chitin deacetylation either through chemical reactions or enzymatic reactions. These compounds can be found in shrimp shells, fish scales, crabs, shellfish, insects, annelids, and some fungal and algal cell walls (Ifa, 2018).

Currently, chitosan from crabs is in great demand because it can reduce cholesterol, uric acid, fat binding, and slimming. Chitosan can reduce LDL cholesterol (Low-Density Lipoprotein) as well as increase the composition of the ratio of HDL (High-Density Lipoprotein) to LDL (Low-Density Lipoprotein). Consequently, Japanese researchers call it an effective hypocholesterolemic agent because it can lower blood cholesterol levels without side effects (El-Sayed, 2021)(Muhammad, 2019). however the discovery of chitosan from squid bone shells is still limited the Effect of Chitosan Bone Powder Squid (Loligo Sp.

Based on the above researchers will conduct research on the effect of chitosan bone powder squid against total cholesterol levels of rats (Rattus norvegicus). This research was conducted based on previous research which proved that squid bones contain chitosan, where chitosan can be used as an anticholesterolemic.

Research methods

Chemical and Reagens

The tools used in this research were Multicheck GCU (Nesco), Syringe injection 3cc (Terumo), glassware (Pyrex), analytical balance (Sartorius). The materials used in this study were aqua destillata, alcohol swabs, acetic acid, hydrochloric acid, Na.CMC, sodium hydroxide, beef tallow, squid bone powder, Simvastatin 10 mg tablet (Fahrenheit, Indonesia) obtained from Makassar Pharmacy.

Experiments

1. Preparation of test animals

   Before preparation of test animals, the research procedure was approved by the Animal Ethics Committee of UNIMERZ No 403.D01.07.091056/I/2022. The test animals used were 12 tails. Initially, adapted experimental animals for 7 days using water, food and laboratory conditions. The animals were placed in a clean-grade animal room at 20-24^{0}C temperature and controlled humidity.

The light and dark cycles were 12 h. He rats were fed a standard diet and water made available ad libitum for this experiment. Afterward, White rats of the wistar strain were used with a body weight of 150 to 200 gs, aged 2 to 4 months, in good physical condition, for several reasons, namely easy to maintain and breed, easy to draw blood and estimated to be physiologically identical with humans (Diarti, 2018). This study used male rats as experimental animals because male rats have a fairly stable hormonal system compared to female rats. In addition, mice are also easier to obtain, easier to handle, and more economical (Suckow MA, 2006).

Rats were grouped into four treatment groups, where each group consisted of three rats. Group I used Na-CMC as a negative control (Na-CMC). Group II used squid bone powder at a dose of 50 mg/kgBB as the 50 mg/KgBB STCC. Group III used squid bone powder at a dose of 100 mg/kgBW as STCC 100 mg/KgBW. And group IV used simvastatin at a dose of 0.18 mg/200 g BW as a positive control. Rats, measurements were carried out three times, namely measuring initial cholesterol levels (K^0), measuring cholesterol levels after being fed fat (K^1), and measuring final cholesterol after treatment (K^2).

2. Sample preparation and processing

Squid bone samples to be tested were obtained from the Antang market, Makassar. 100 gs of squid bones were washed, dried in the sun not exposed to direct sunlight, mashed using grinding and sieved through a 60 mesh sieve (Yulianis, 2020).

3. Making chitosan from squid bone powder

The internal shell of the squid is dried, then crushed using a mortar and pestle until it becomes a powder. Deproteination process. The powder was cooked with 1 N NaOH at 90°C for 60 minutes. Then filtered and neutralized with distilled water. The demineralization process at a temperature of 100°C using 1 N HCl solution while stirring constantly for 120 minutes. After that it is cooled, filtered and neutralized with distilled water. The product of this process is called chitin. Chitin was then added to a NaOH solution with a concentration of 50% at a temperature of 90°C while stirring constantly for 60 minutes in the deacetylation process. The result is filtered and then washed with distilled water until the pH is neutral. The result is called chitosan (Rochmawati & Nabila dkk, 2018).

4. Preparation of 5% and 10% chitosan

Chitosan is made by weighing the chitosan powder according to the desired concentration. For concentrations of 5% and 10%, 5 gs and 10 gs were weighed and then dispersed in 100 ml Na-CMC (1%w/v) (Fawwaz, 2013).

5. Preparation of Na-CMC suspension

Na-CMC 1% solution was prepared by dissolving 1 g of Na-CMC in sufficient hot water while stirring to form a colloidal solution. After that, it was filled with distilled water up to 100 ml (Yulianis, 2020).

6. Preparation of simvastatin suspension

The simvastatin tablet is crushed until smooth, then removed. Weighed 0.5 gs of Na-CMC, sprinkled on 7.5 mL of warm aquadest, ground until homogeneous. Simvastatin powder was added slowly and ground until homogeneous. Diluted with 10 mL of distilled water. Put in a container. Added aquadest to 100 mL. Simvastatin dose 0.18mg/200g BW rats (Nyoman, 2014).

7. Preparation for hypercholesteremia in experimental rats

The high-fat diet feed used consisted of 10% beef fat, 20% used cooking oil, and 20% quail egg yolk mixed in 120 ml for each rat given orally. A high-fat diet is made by heating solid beef fat to obtain a liquid form (beef fat oil), then mixed with beef oil, cooking oil, quail egg yolk and stirred quickly until an emulsion corpus is formed, which is then added with water up to a volume of 120 ml. into the fixed emulsion body while stirring rapidly to form a smooth emulsion. The high-fat diet was given twice, namely 08.00 at the time before eating and 17.00 at night. This induction feed is made fresh every day and given as soon as possible to avoid clumping of beef fat oil (Gunawan, 2018).

8. Induction of squid bone chitosan powder

The experimental animals white rats wistar strain were weighed each to determine how many doses to be given. White rats that had hypercholesterolemia were given a suspension for 7 days by weighing according to the body weight of the experimental animals. The administration was carried out once with a volume of 5 ml given to experimental white rats by disonde or oral (the result of the administration was the conversion of each white rat's body weight). After being given successively, the cholesterol levels will be checked again (Diarti, 2018).

9. Blood sampling of rats

Rats whose blood samples will be taken are fasted for 10 to 12 hours. The rat's tail was cleaned with cotton that had been given 70% alcohol so that the dirt on the tail could be lifted. Next, a few drops of blood are taken from the tail that has been cut, then the blood is dripped on the cholesterol strip (Tubagus, 2015)(Gunawan, 2018).

10. Examination of total blood cholesterol levels of rats

    The blood of the experimental animal was taken white rat wistar strain through capillary blood vessels by cutting the end of the tail of the experimental animal aseptically while gently massaging from the hip to the tip of the tail. Experimental animals were fasted for 10 to 12 hours. Before checking the total cholesterol level, drinking water was still given. The total cholesterol level of male white rats was measured in the morning using the Easy Touch GCU stick method. The normal value of white rats wistar strain is 40 to 130 mg/dl, if the blood concentration of the experimental animals increases to 20%, it can be said that the experimental animals have hypercholesterolemia. Performed before and after the treatment (Diarti, 2018).

Data Analysis

The data obtained were analyzed using the One Way Anova statistical test with a 95% confidence level. Used to determine the existence of a significant difference between treatments in reducing total blood cholesterol levels.

Result and Discussion

The purpose of this study was to determine whether squid bone powder had an effect on reducing total cholesterol levels in rats using parameters of decreasing cholesterol levels before and after administration of squid bone powder. This study used Na-CMC as a negative control and simvastatin as a positive control. Cholesterol is a fatty substance circulating in the blood, yellowish in color and shaped like wax, which is produced by the liver and is needed by the body. Cholesterol is a lipid that is not hydrolyzed and is the main sterol in human tissues. Cholesterol plays an important role in plasma lipoproteins and plasma membranes and is the precursor of a large number of steroid compounds (Diarti, 2018).

Bones in squid can be used as a source of medicinal ingredients, one of which is chitosan. Chitin was obtained by purification using bone powder in squid to be deproteinated with 3.5% NaOH and demineralized with 1N HCl (Yulianis, 2020).

The method used to produce chitosan from squid bones. There are three processes. The first is the deproteinase process to remove the protein contained in the bones of squid, where the simplicia that has been weighed is then mixed with 3.5% NaOH and then boiled using a water bath for 60 minutes at 90°C while occasionally stirring then cooled and filtered. The second is the demineralization process to remove the minerals contained in the bones of the squid, in which the simplicia resulting from the deproteinase process is mixed again with 1 N HCl and then boiled using a water bath for 120 minutes at 100°C while stirring occasionally, then cooled and filtered. The third is the deacetylation process to convert chitin into chitosan, where the simplicia that has been weighed is then mixed with 50% NaOH and then boiled using a water bath for 60 minutes at 90°C while occasionally stirring, then cooled and filtered to obtain chitosan (Khan, T. A., 2002). The chitosan obtained was then subjected to a qualitative test to see whether the chitosan obtained from this study was of sufficient standard, by dissolving the chitosan with 2% acetic acid (Sry Agustina, I Made Dira Swantara, 2015).

In addition, this study also used simvastatin tablets as a positive control. Simvastatin is a reductase-inhibiting compound (HMG-CoA-reductase-inhibitors) which is able to reduce endogenous cholesterol synthesis in the liver and thus there is a strong decrease in total cholesterol, LDL (by 30 to 40%), TG and VLDL more mildly , while HDL is raised (Hoan, 2007). Positive control is used to obtain a clearer picture of the reduction in cholesterol levels.

In this regard, the researcher argued that measurements were not carried out every day so that the mice's condition was maintained. If measurements are taken every day, the rats will experience stress which will affect the rat's condition (Suckow MA, 2006). Based on this, time-lapse measurements are carried out so that the rats can provide fairly accurate data.

Fat feeding was carried out for 7 days as much as 20 gs/rat to increase the total cholesterol level of the rats (hypercholesterolemia). Mice are said to be hypercholesterolemia if their total blood cholesterol level is > 200 mg/dL (Putri, 2018). From day 0 (K^0) to day 7 (K^1), the rats experienced an increase in cholesterol levels which varied quite a bit in each group. Each group of rats had total cholesterol levels > 200 mg/dL (Table 1.) Feeding hypercholesterolemia containing 10% beef fat, 20% used cooking oil, and 20% quail egg yolk increased the total cholesterol levels of rats in this study. Egg yolk and beef fat are sources of animal cholesterol and fat which can increase total cholesterol and triglyceride levels in the blood (Murray,R.K., 2003). On day 7 (K^1) to day 14 (K^2), the negative control group did not decrease cholesterol levels of rats, this was due to Na-CMC does not have active ingredients that can lower cholesterol levels.

Tabel 1. Cholesterol levels in male rats were given a suspension of squid bone powder.

Group	Treatment	Cholesterol level (mg/dL)			P-value
		K0	K1	K2	
1	Negative control (Na-CMC)	186±1.13	234,7±1.04	276,6±1.11	0.917
2	STCC* 50 mg/kgBB	177±2.34	247±2.13	148,6±1.36	0.543
3	STCC* 100 mg/kgBB	190±1.21	252±1.31	121,3±2.05	0.943
4	Positive control (Simvastatin)	184±1.30	231±1.54	112,6±1.03	0.774

*STCC: Squid Bone Powder; P-Value : 0.641 (P>0.005)

In group II there was a slight decrease in cholesterol levels, while in group III there was a very clear decrease in cholesterol levels close to the initial cholesterol level. The similar response was observed in the positive control group.

On the other hand, on the 14th day (K2) after treatment, there was a significant difference in total cholesterol levels for each group. The negative control group I using CMC Na showed an increase in total cholesterol levels. The average total cholesterol level on the 7th day of high-fat diet (K^1) slightly increased by around 25% or around 234 mg/dL. whereas on day 14 (K^2) there was an increase of around 48% or 276.6 mg/dL from the initial total cholesterol. In group II, administration of 50 mg/kg BW of squid bone powder showed a decrease in total cholesterol levels of rats. The average total cholesterol level on day 14 (K^2) decreased by around 40% or 148.6 mg/dL from total cholesterol on day 7 (K^1). In group III, 100 mg/kg BW of squid bone powder (Loligo sp.) showed a decrease in total cholesterol levels in rats. The average total cholesterol level on day 14 (K^2) decreased by around 52% or 121.3 mg/dL from total cholesterol on day 7 (K^1). In group III, administration of 0.18 mg/kg of Simvastatin showed a decrease in total cholesterol levels of rats. The average total cholesterol level on day 14 (K^2) decreased by around 51% or 112.6 mg/dL from total cholesterol on day 7 (K^1). Based on the results of the one way test analysis, it showed that the data on total cholesterol levels in each group had an average that was not significantly different in each group from a significant value which indicated a value of P = 0.641 (P> 0.005). This means that the value of reducing cholesterol before and after feeding Squid bone powder to mice is not significant.

Conclusion

Based on the research that has been done, it can be concluded that giving squid bone powder to hypercholesterolemic rats can reduce total blood cholesterol levels. Squid bone powder at a dose of 100 mg/Kg BW reduced it by up to 52% but there was no significant difference compared to the control group (P>0.005).

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