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Original Article

Effect of *Lissachatina fulica* chitosan on the antioxidant and lipid profile of hypercholesterolemic male Wistar rats

[Efecto del quitosano de *Lissachatina fulica* sobre el perfil antioxidante y lipídico de ratas Wistar macho hipercolesterolémicos]

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Abstract

Context: The increase in reactive oxygen species production in hypercholesterolemia can degrade polyunsaturated fatty acids and form malondialdehyde (MDA). There is a need for a natural alternative treatment, such as *Lissachatina fulica* chitosan.

Aims: To analyze the potential of *Lissachatina fulica* chitosan in increasing the superoxide dismutase (SOD) level, reducing lipid profile, and MDA level of male Wistar rats with hypercholesterolemia model.

Methods: The male Wistar rats were divided into six groups (n = 4): P0 (normal control); P1-P5 fed a high-fat diet for four weeks. P1 were treated with fed with a high-fat diet; P2 were treated with ezetimibe of 0.18 mg/200 g BW; P3, P4, and P5 Groups; fed with a high-fat diet and *Lissachatina fulica* chitosan of 100 mg, 200 mg, and 300 mg/200g BW, respectively. The lipid profile was conducted in rat blood samples by using the CHOP-PAP method, the MDA analysis using the TBARS test, and SOD assay analysis using a kit.

Results: The results reveal that the treatment *Lissachatina fulica* chitosan (300 mg/200 g BW) was significantly effective ($p<0.05$) to decrease in total cholesterol level (96.7 ± 1.9 mg/dL), triglyceride (75.6 ± 1.6 mg/dL), LDL (29.8 ± 2.5 mg/dL), and MDA (1.2 ± 0.1 nmol/mL) as well as a significant increase ($p<0.05$) in HDL level (75.0 ± 1.7 mg/dL) and SOD (74.6 ± 2.1 unit/mL).

Conclusions: *Lissachatina fulica* chitosan can reduce total cholesterol, triglyceride, LDL, and MDA, and increase HDL and SOD levels.

Keywords: hypercholesterolemia; lipid profile; *Lissachatina fulica* chitosan; MDA; SOD.

Resumen

Contexto: El aumento en la producción de especies reactivas de oxígeno en la hipercolesterolemia puede degradar los ácidos grasos poliinsaturados y formar malondialdehído (MDA). Existe la necesidad de un tratamiento alternativo natural, como el quitosano de *Lissachatina fulica*.

Objetivos: Analizar el potencial del quitosano de *Lissachatina fulica* para aumentar el nivel de superóxido dismutasa (SOD), reducir el perfil lipídico y el nivel de MDA de ratas Wistar macho en un modelo de hipercolesterolemia.

Métodos: Las ratas Wistar macho se dividieron en seis grupos (n = 4): P0 (control normal); P1-P5 alimentados con una dieta rica en grasas durante cuatro semanas. P1 fueron tratados con alimentación con una dieta rica en grasas; P2 fueron tratados con ezetimiba de 0,18 mg/200 g de peso corporal; Grupos P3, P4 y P5; alimentados con una dieta rica en grasas y quitosano de Lissachatina fulica de 100 mg, 200 mg y 300 mg/200 g de peso corporal, respectivamente. El perfil de lípidos se realizó en muestras de sangre de rata utilizando el método CHOP-PAP, el análisis MDA utilizando la prueba TBARS y el análisis de ensayo SOD utilizando un kit.

Resultados: Los resultados revelan que el tratamiento de quitosano de *Lissachatina fulica* (300 mg/200 g BW) fue significativamente efectivo ($p<0,05$) para disminuir el nivel de colesterol total (96,7 ± 1,9 mg/dL), triglicéridos (75,6 ± 1,6 mg/dL), LDL (29,8 ± 2,5 mg/dL) y MDA (1,2 ± 0,1 nmol/mL), así como un aumento significativo ($p<0,05$) en el nivel de HDL (75,0 ± 1,7 mg/dL) y SOD (74,6 ± 2,1 unidades/mL).

Conclusiones: El quitosano de *Lissachatina fulica* puede reducir el colesterol total, los triglicéridos, LDL y MDA, y aumentar los niveles de HDL y SOD.

Palabras Clave: hipercolesterolemia; *Lissachatina fulica* quitosano; MDA; perfil lipídico; SOD.

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INTRODUCTION

Hypercholesterolemia refers to a condition of an increase in total cholesterol level, LDL, or triglyceride, and a decrease in HDL level, which is defined as a lipid metabolism disorder (Ibrahim et al., 2020; Wells et al., 2015). Hypercholesterolemia is indicated by an increase in cholesterol level ≥240 mg/dL (Bunton et al., 2011; He et al., 2004; Koo et al., 2021; Yang et al., 2020). Several recent studies investigated that high-cholesterol diets cause an increase in total cholesterol and an LDL level of blood that lead to blood vessel wall thickening, known as atherosclerosis and cardiovascular (Avci et al., 2006; Mannarino et al., 2009; Wahjuni, 2014; Wang et al., 2017). Atherosclerosis and cardiovascular disease have become leading causes of morbidity and mortality in Indonesia (Hussain et al., 2016; Maharani and Tampubolon, 2014; Maharani et al., 2019).

High cholesterol increases oxidative stress (OS) and nicotinamide adenine dinucleotide phosphate (NADPH oxidase) activity, known as NOX, which is responsible for reactive oxygen species (ROS) generation. Meanwhile, NOX1 (NADPH oxidase 1) and NOX2 oxidase are the primary sources of ROS in the hypercholesterolemic artery walls, so they take part in oxidative stress causing endothelium dysfunction and inflammation in blood vessels (Canugovi et al., 2019; Drummond et al., 2011; Magnani and Mattevi, 2019; Sedeek et al., 2009). The increase in ROS production can degrade polyunsaturated fatty acids (PUFA) and form malondialdehyde (MDA) (Ayala et al., 2014; Kurutas, 2016). The MDA level increases in Wistar rats under hypercholesterolemic conditions (Aryanugraha et al., 2012; Valko et al., 2007; Wahjuni, 2014; Widhiantara et al., 2021). MDA is the final product of the lipid peroxidation process and can cause free compounds or bonds to tissues (Aryanugraha et al., 2012; Michalski et al., 2008; Yui et al., 2021; Yulianti et al., 2020). High plasma MDA indicates an increase in free radical activity and a decrease in superoxide dismutase (SOD) enzyme (Lecumberri et al., 2007; Wahjuni, 2014). SOD, catalase, glutathione peroxide (GPX), and non-enzyme antioxidant constitute intracellular antioxidants with a defense mechanism against tissue damage due to the formation of free radicals (He et al., 2004; 2012; Ighodaro and Akinloye, 2018; Yang et al., 2008; Yasmeen and Hasnain, 2015). On the contrary, NADPH oxidase forms ROS as the only function. They are found in various tissues and organs, and they also play a crucial role in causing disorders related to oxidative stress and affect molecular and cellular (Camiletti-Móiron et al., 2015; Kawamura and Muraoka, 2018; Yang et al., 2008).

An antioxidant is essential to balance oxidants and free radicals, which is beneficial for hypercholesterolemia (Halliwell, 2012). However, the amount of endogenous antioxidants inside the body is limited in preventing free radicals. Therefore, the body cannot inhibit oxidative stress, and endogenous antioxidant is required (Ayunda et al., 2019). Chitosan from snail shells becomes one of the sources of exogenous antioxidants (Umarudin et al., 2019). Exogenous antioxidants balance the oxidant/ antioxidant system due to the limited number of endogenous antioxidant systems to neutralize ROS (Koçyiğit, 2016). Antioxidants from plants have been commonly studied, but animal antioxidants have not been studied widely. Therefore, animal materials are significant to be utilized in hypercholesterol therapy. Moreover, these materials have no side effects compared to synthetic drugs.

In Indonesia, snails are used only for meat and mucus, while the shells are discarded as waste. *Lissachatina fulica* (snail shells) contain a high CaO (calcium oxide) of 88-99% (Puspitasari et al., 2021; Vanitha et al., 2017). *Lissachatina fulica* (Bowdich, 1822) does not have a poison (toxic) as in snakes or other poisonous animals (Nurinsiyah and Hausdorf, 2019). The shell morphology of *Lissachatina fulica* is yellow or light brown with dark brown vertical irregular lines decorated. The mouth of the shell is oval but relatively wide. Apex or pointed apex of the shell (Benthem-Jutting, 1952; Nurinsiyah and Hausdorf, 2019).

Snail shells also contain 67.16% chitosan (Maya et al., 2020; Sundalian, 2021). Chitosan [β-(1-4)-N-acetyl-D-glucosamine] belongs to the amino polysaccharide group, a cationic natural biopolymer with unique characteristics, and is used in the biomedical industry. One of the benefits is for biomedical drug delivery, and also can be used for the pharmaceutical industry (Brunner et al., 2009; El-Naggar et al., 2022; Pillai et al., 2009; Wang et al., 2017; Yen and Mau, 2007). Thus, this research is critical because there has not been any information about chitosan from snail shells. It is because most chitosan is sourced from shrimp and lobsters. Meanwhile, chitosan produced by snail shells has not been widely used yet. The findings of this research aim to prove the claim of chitosan from snail shells (*Lissachatina fulica*) as an anti-hypercholesterol. This research aims to reveal the potential of *Lissachatina fulica* chitosan on male rats fed with high cholesterol diets by measuring the lipid profile (total cholesterol, LDL, triglyceride, LDL, and HDL), MDA, and SOD level.

MATERIAL AND METHODS

Materials

Snail shell samples (*Lissachatina fulica*) were collected from Ketami village, Kediri district, East Java, Indonesia (7°50′03.8″S 112°03′21.9″E). The snail specification was carried out at the National Research and Innovation Agency (BRIN) with Document ID number FR-ZO-07-04 on January 1, 2021.

All reagents included HCL (Merck, Hohenbrun, Germany), distiller water, NaOH (Merck, Hohenbrun, Germany), NaOCl (Merck, Hohenbrun, Germany), ezetimibe (Organom Pharma Indonesia, distilled water, LDL precipitant (DiaSys, Holzheim, Germany), triglycerides and cholesterol FS (DiaSys, Holzheim, Germany), MDA assay kit (Bioassay System, Kampenhout, Belgium), and SOD assay kit Catalog No: ESOD-100 (Bioassay System, Hayward, CA 94545, USA).

Experimental animals

Twenty-four (24) male Wistar rats weighing 180-200 g were placed in individual cages from the CFNS of UGM Rat House Experimental Laboratory. During the experiment, Wistar rats were *ad libitum* fed with BR-II pellets and ordinary water. The experimental rats in this study were treated according to animal use guidelines (Animal Care Program, 2011), and these experimental animals were approved by the research ethics commission (Animal Care and Use Committee) of the University of Brawijaya, Indonesia (Approval No. 112-KEP-UB-2021) on 8 October 2021.

Chitosan snail shell isolation

Snail shell waste used in this study was soaked in warm water for 30 minutes and then washed under running water until it got clean. The shells were dried under direct sunlight, then crushed and strained using a 100-mesh strainer. Afterward, chitosan was isolated from snail shells in four stages: demineralization, deproteination, depigmentation, and deacetylation, each of which was stirred on a hot plate magnetic stirrer at a temperature of 90- 100°C. At the demineralization stage, 10 g of snail shell powder was stirred in HCl 6% 1 L (10:1 v/w) solution at 90°C and 400 rpm for six hours until it formed foam. After that, the results were strained, and the solid form was neutralized with distilled water and heated in the oven at 100°C for three hours or until it resulted in constant weight. Further, at the deproteination stage, the solid form resulting from the demineralization process was stirred in NaOH 4% (10:1 v/w) for six hours at 100°C and 400 rpm. Subsequently, the material was strained and neutralized with distilled water. The solid form was then dried in the oven at 100°C for three hours or until the constant weight was obtained. At the depigmentation stage, the solid form, the result of the deproteination stage, was stirred in NaOCl 0.315% (10:1 v/w) at 90°C and 400 rpm for six hours, then strained and neutralized with distilled water. The solid form resulting from the neutralization process was dried in the oven at 95°C for three hours or until the constant weight was obtained. Moreover, at the deacetylation stage, the solid form produced by the depigmentation process was stirred in NaOH 70% (10:1 v/w) for six hours at 120°C and 400 rpm, then strained and neutralized with distilled water. The solid form was then dried in the oven at 100°C for three hours or until it resulted in constant weight.

Preparation for the hypercholesterolemia model and treatment using *Lissachatina fulica* chitosan

The *Lissachatina fulica* chitosan was tested *in vivo* on male Wistar rats with a body weight of about 200 g, 2-3 months of age, healthy, clear-red eye morphology, non-standing hair, and agile. In this study, 24 rats were involved. They were previously acclimatized and randomly divided into six groups (four rats for each group). The rats were given *ad libitum* feeding and water. The groups consisted of the P0 Group: normal control; P1 Group: fed with a high-fat diet for four weeks; P2 Group: fed with a high-fat diet and ezetimibe 0.18 mg/200 g BW; P3, P4, and P5 Groups: fed with a high-fat diet and *Lissachatina fulica* chitosan of 100 mg, 200 mg, and 300 mg/200 g BW, respectively. The treatment was conducted by giving chitosan (dissolved in distilled water) orally for 30 days. The lipid profile (total cholesterol, triglyceride, HDL-cholesterol, and LDC-cholesterol), MDA, and SOD were measured on days 0, 21, and 31. Rats fasted for 14 hours before their blood was collected. The procedure is illustrated in Fig. 1.

In the schematic of Fig. 1A, the rats were grouped, and in Fig. 2B, the rats were acclimatized for seven days. On the 8th day, blood sampling was taken from all groups to measure the lipid profile levels (total cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol). The P1-P5 group of rats was treated with a high-fat diet for four weeks, except for Group P0 as the normal control group, followed by blood sampling to measure the lipid profile level (total cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol). Each group was given a different treatment, according to Fig. 1A. On the 21st day, the side blood was measured for lipid profile levels and continued to be treated until the 30th day. On the 31st day, blood sampling was conducted to measure the lipid profile levels (total cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol), MDA, and SOD levels.

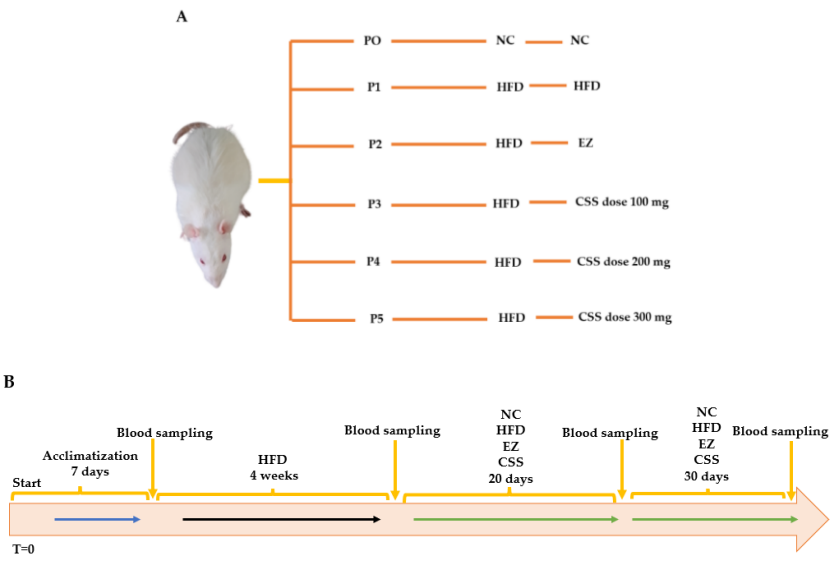

Figure 1. Scheme of **(A)** Animal grouping, and **(B)** Representation of experimental protocols.
NC: Normal control; HFD: high-fat diet; EZ: Ezetimibe; CSS: *Lissachatina fulica* chitosan.

The lipid profile (total cholesterol, LDL, triglycerides, and HDL) analysis

The rats' blood was taken through the orbital sinus and accommodated in the tube. The blood was left for 15 min and centrifuged for 10 min at 4000 rpm. The lipid profile parameters (total cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol) were measured with the CHOP-PAP enzyme method (Ayunda et al., 2019).

MDA and SOD level analysis

The blood was collected in a microtube and centrifuged at 4000 rpm for 10 min at room temperature; the plasma was separated and then put in a microtube of 1 mL and stored in a cooler at -70°C in the dark. The MDA serum was measured using the thiobarbituric acid reactive test method (TBARS) (Sun and Zigman, 1978) and SOD level based on with SOD Assay Kit (Catalog No: ESOD-100, Bioassay System) (Widhiantara et al., 2021).

Statistical analysis

The statistical data analysis was performed utilizing SPSS software version 13.0. Lipid profile parameters were analyzed with two-way ANOVA, while MDA and SOD were analyzed with one-way ANOVA. If the result was significant, the data was further tested with LSD with Tukey's test. P-values <0.05 were statistically significant.

RESULTS

In Table 1, the total cholesterol level of Group P0 amounted to 87.4 ± 3.9 mg/dL, considered a normal level. In Group P1, the total cholesterol level reached 197.2 ± 3.7 mg/dL, which is considered hypercholesterolemia. Hypercholesterolemic rats had total cholesterol >140 mg/dL (Hirunpanich et al., 2005). The decrease of cholesterol level in Group P2 given by ezetimibe was 106.1 ± 3.5 mg/dL, and the lowest was in the groups with *Lissachatina fulica* chitosan treatment: P5, P4, and P3 by 96.7 ± 1.9, 101.3 ± 2.8, and 120.4 ± 3.7 mg/dL, respectively. The chitosan treatment reduced total cholesterol levels, which were significantly different from P0, P1, and P2 ($p<0.05$). Groups P4 and P5 were not significantly different ($p>0.05$), and P4 was not significantly different from P3 ($p>0.05$).

The average level of the triglyceride of Group P0 was 66.6 ± 2.6 mg/dL, meaning that the triglyceride level in rats was normal (Table 2). Group P1 had triglyceride level of 132.6 ± 8.3 mg/dL. However, the level was two times higher than P0. The triglyceride level of Group P2 was 81.6 ± 3.9, significantly different from P0, P1, P2, P3, and P5 ($p<0.05$) and not significantly different from P4 ($p>0.05$). The lowest triglyceride level was in chitosan treatment groups, i.e., P5, P4, and P3, by 75.6 ± 1.7, 80 ± 3.1, and 101.0 ± 3.5 mg/dL, respectively. Groups P3, P4, and P5 had low triglyceride levels and significantly differed from P1 ($p<0.05$). Groups P4 and P5 were not significantly different from P2 ($p>0.05$).

Table 1. Total cholesterol level (mg/dL) after treatments using various dosages of *Lissachatina fulica* chitosan.

Time/group	Before hypercholesterolemic diet	After hypercholesterolemic diet	After treatments *Lissachatina fulica* chitosan	
			20th-day treatment	30th-day treatment
P0	$84.1 \pm 2.8^{a,1}$	$84.8 \pm 2.9^{a,2}$	$86.2 \pm 3.3^{a,3}$	$87.4 \pm 3.9^{a,4}$
P1	$83.5 \pm 3.0^{b,1}$	$194.8 \pm 3.3^{b,2}$	$195.9 \pm 3.2^{b,3}$	$197.2 \pm 3.7^{b,4}$
P2	$83.5 \pm 2.6^{c,1}$	$191.2 \pm 3.7^{c,2}$	$126.9 \pm 3.9^{c,3}$	$106.1 \pm 3.5^{c,4}$
P3	$84.5 \pm 2.3^{d,1}$	$190.1 \pm 4.5^{d,2}$	$141.9 \pm 4.3^{d,3}$	$120.4 \pm 3.7^{d,4}$
P4	$82.0 \pm 2.9^{c,e,1}$	$190.7 \pm 4.2^{c,e,2}$	$125.4 \pm 3.0^{c,e,3}$	$101.3 \pm 2.8^{c,e,4}$
P5	$83.7 \pm 1.8^{e,1}$	$193.2 \pm 1.9^{e,2}$	$121.1 \pm 2.8^{e,3}$	$96.7 \pm 1.9^{e,4}$

Numbers followed by the same letters in the same column do not show any significant differences between groups based on the Tukey test at the 95% confidence level. Numbers followed by the same letters in the same row do not show any significant differences between times based on the Tukey test at the 95% confidence level. Data represent mean ± SD (n = 24). P0: normal control; P: high-fat diet; P2: ezetimibe 0.18 mg/200g BW; P3: *Lissachatina fulica* chitosan of 100 mg/200g BW; P4: *Lissachatina fulica* chitosan of 200 mg/200g BW; and P5: *Lissachatina fulica* chitosan of 300 mg/200g BW.

Table 2. Triglyceride level (mg/dL) after treatments using various dosages of *Lissachatina fulica* chitosan.

Time/group	Before hypercholesterolemic diet	After hypercholesterolemic diet	After treatments *Lissachatina fulica* chitosan	
			20th-day treatment	30th-day treatment
P0	$64.3 \pm 3.4^{a,1}$	$64.9 \pm 3.3^{b,2}$	$65.8 \pm 2.9^{a,3}$	$66.6 \pm 2.6^{a,4}$
P1	$61.9 \pm 1.2^{b,1}$	$129.5 \pm 8.4^{b,2}$	$130.6 \pm 8.7^{b,3}$	$132.6 \pm 8.3^{b,4}$
P2	$64.8 \pm 1.9^{c.1}$	$116.8 \pm 5.2^{c,2}$	$83.3 \pm 3.8^{c,3}$	$81.6 \pm 3.9^{c,4}$
P3	$64.8 \pm 2.8^{d,1}$	$129.0 \pm 2.8^{d,2}$	$103.2 \pm 3.3^{d,3}$	$101.0 \pm 3.5^{d,4}$
P4	$63.9 \pm 1.2^{c,e,1}$	$126.8 \pm 3.8^{c,e,2}$	$84.0 \pm 3.2^{c,e,3}$	$80.0 \pm 3.1^{c,e,3}$
P5	$62.2 \pm 1.4^{c,f,1}$	$124.1 \pm 1.7^{c,f,2}$	$80.1 \pm 1.9^{c,f,3}$	$75.6 \pm 1.7^{c,f,3}$

Numbers followed by the same letter in the same column do not show any significant differences between groups based on the Tukey test at the 95% confidence level. Numbers followed by the same letter in the same line do not show any significant differences between times based on the Tukey test at the 95% confidence level. Data represent mean ± SD (n = 24). P0: normal control; P: high-fat diet; P2: ezetimibe 0.18 mg/200 g BW; P3: *Lissachatina fulica* chitosan of 100 mg/200 g BW; P4: *Lissachatina fulica* chitosan of 200 mg/200 g BW; and P5: *Lissachatina fulica* chitosan of 300 mg/200 g BW.

The LDL level of Group P1 was 86.7 ± 0.9 mg/dL, which was three times higher than P0 (Table 3). The average LDL level of Group P0 was 26.8 ± 1.3 mg/dL, meaning that the LDL level in rats was normal. The LDL level of Group P2 was 35.3 ± 3.6 mg/dL, significantly different from P0, P1, P2, P3, and P5 ($p<0.05$). The lowest decrease in LDL level was in the chitosan treatment groups, namely P5, P4, and P3, by 29.9 ± 2.5, 37.1 ± 2.0, and 54.1 ± 3.2 mg/dL, respectively. The chitosan given to Groups P3, P4, and P5 lowered LDL levels and significantly differed from P0, P1, and P2 ($p<0.05$).

The average HDL level of Group P0 was 82.8 ± 1.5 mg/dL, meaning that the HDL level in rats was normal (Table 4). Group P1 had an HDL level of 20.7 ± 1.4 mg/dL. The level was four times lower than P0. The HDL level of Group P2 given by ezetimibe was 69.1 ± 2.0 mg/dL, significantly different from P3 and P5 ($p<0.05$) and not significantly different from P4 ($p<0.05$). The highest increase in HDL levels was in groups P5, P4, and P3 by 75.0 ± 1.7, 70.29 ± 2.0, and 63.9 ± 2.4 mg/dL, respectively. The HDL levels of P5, P4, and P3 were significantly different from P1 ($p<0.05$).

The average level of MDA of Group P0 was 1.3 ± 0.2 nmol/mL, and Group P1 had an MDA level of 10.0 ± 0.2 nmol/mL (Table 5). The level was eight times higher than P0. The decrease in the MDA level of Group P2 treated with ezetimibe was 2.1 ± 0.3 nmol/mL, significantly different from P3 and P5 ($p<0.05$) and not significantly different from P4 ($p<0.05$). The lowest decrease in MDA levels was in Groups P5, P4, and P3 by 1.2 ± 0.1, 2.3 ± 0.5, and 5.6 ± 0.4 nmol/mL, respectively. However, Groups P5, P4, and P3 significantly differed from P1 ($p<0.05$).

The average SOD level in Group P0 was 82.3 ± 3.63 unit/mL, and Group P1 had a SOD level of 29.5 ± 2.9 unit/mL, three times lower than Group P0. The increase in the SOD level of Group P2 was 74.2 ± 2.8 unit/mL, significantly different from Group P3 ($p<0.05$) and slightly different from Groups P4 and P5 ($p<0.05$). The highest increase in the SOD levels was in Groups P5, P4, and P3 by 74.6±2.1, 63.1±2.1, and 41.8 ± 4.3 unit/mL, respectively. The P5, P4, and P3 Groups significantly differed from Group P1 ($p<0.05$).

Table 3. LDL level (mg/dL) after treatments using various dosages of *Lissachatina fulica* chitosan.

Time/group	Before hypercholesterolemic diet	After hypercholesterolemic diet	After treatments *Lissachatina fulica* chitosan	
			20th-day treatment	30th-day treatment
P0	$23.2 \pm 0.9^{a,1}$	$24.1 \pm 0.9^{a,2}$	$26.1 \pm 0.9^{a,3}$	$26.8 \pm 1.3^{a,4}$
P1	$23.2 \pm 1.7^{b,1}$	$83.2 \pm 1.1^{b,2}$	$84.2 \pm 0.9^{b,3}$	$86.7 \pm 0.9^{b,4}$
P2	$24.5 \pm 1.5^{c,1}$	$77.3 \pm 2.9^{c,2}$	$55.5 \pm 3.4^{c,3}$	$35.3 \pm 3.6^{c,4}$
P3	$25.0 \pm 1.6^{d,1}$	$84.9 \pm 3.4^{d,2}$	$71.1 \pm 3.3^{d,3}$	$54.1 \pm 3.2^{d,4}$
P4	$24.4 \pm 2.3^{e,1}$	$84.2 \pm 1.7^{e,2}$	$54.0 \pm 2.1^{e,3}$	$37.1 \pm 2.0^{e,4}$
P5	$25.4 \pm 1.4^{f,1}$	$80.7 \pm 2.1^{f,2}$	$46.9 \pm 2.3^{f,3}$	$29.9 \pm 2.5^{f,4}$

Numbers followed by the same letter in the same column do not show any significant differences between groups based on the Tukey test at the 95% confidence level. Numbers followed by the same letter in the same line do not show any significant differences between times based on the Tukey test at the 95% confidence level. Data represent mean ± SD (n = 24). P0: normal control; P: high-fat diet; P2: ezetimibe 0.18 mg/200 g BW; P3: *Lissachatina fulica* chitosan of 100 mg/200 g BW; P4: *Lissachatina fulica* chitosan of 200 mg/200 g BW; and P5: *Lissachatina fulica* chitosan of 300 mg/200 g BW.

Table 4. HDL level (mg/dL) after treatments using various dosages of *Lissachatina fulica* chitosan.

Time/group	Before hypercholesterolemic diet	After hypercholesterolemic diet	After treatments *Lissachatina fulica* chitosan	
			20th-day treatment	30th-day treatment
P0	$85.0 \pm 1.5^{a,1}$	$86.1 \pm 1.9^{a,2}$	$84.5 \pm 1.9^{a,3}$	$82.8 \pm 1.5^{a,4}$
P1	$86.2 \pm 3.3^{b,1}$	$22.4 \pm 1.4^{b,2}$	$21.5 \pm 1.8^{b,3}$	$20.7 \pm 1.4^{b,4}$
P2	$87.0 \pm 1.5^{c,1}$	$23.4 \pm 1.4^{c,2}$	$67.5 \pm 1.5^{c,3}$	$69.0 \pm 2.0^{c,4}$
P3	$85.0 \pm 2.3^{d,1}$	$23.1 \pm 2.1^{d,2}$	$59.6 \pm 2.2^{d,3}$	$63.9 \pm 2.4^{d,4}$
P4	$85.3 \pm 3.0^{c,e,1}$	$23.4 \pm 1.4^{c,e,2}$	$67.5 \pm 1.5^{c,e,3}$	$70.3 \pm 2.0^{c,e,4}$
P5	$83.5 \pm 3.0^{f,1}$	$23.6 \pm 1.1^{f,2}$	$71.8 \pm 1.5^{f,3}$	$75.0 \pm 1.7^{f,4}$

Numbers followed by the same letters in the same column do not show significant differences between groups based on the Tukey test at the 95% confidence level. Numbers followed by the same letters in the same row do not show significant differences between times based on the Tukey test at the 95% confidence level. Data represent mean ± SD (n = 24). P0: normal control; P: high-fat diet; P2: ezetimibe 0.18 mg/200 g BW; P3: *Lissachatina fulica* chitosan of 100 mg/200 g BW; P4: *Lissachatina fulica* chitosan of 200 mg/200 g BW; and P5: *Lissachatina fulica* chitosan of 300 mg/200 g BW.

Table 5. MDA and SOD levels after treatments using various dosages of *Lissachatina fulica* chitosan.

Group	MDA (nmol/mL)	SOD (unit/mL)
P0	$1.3 \pm 0.2^{a,f}$	82.4 ± 3.6^{a}
P1	10.0 ± 0.2^{b}	29.5 ± 2.9^{b}
P2	2.1 ± 0.3^{c}	74.2 ± 2.8^{c}
P3	5.6 ± 0.4^{d}	41.8 ± 4.3^{d}
P4	$2.3 \pm 0.5^{a,c,e}$	$63.1 \pm 2.1^{c,e}$
P5	1.2 ± 0.1^{f}	$74.6 \pm 2.1^{c,f}$

Numbers followed by the same letters in the same column do not show significant differences between groups based on the Tukey test at the 95% confidence level. Data represent mean ± SD (n = 24). P0: normal control; P: high-fat diet; P2: ezetimibe 0.18 mg/200 g BW; P3: *Lissachatina fulica* chitosan of 100 mg/200 g BW; P4: *Lissachatina fulica* chitosan of 200 mg/200 g BW; and P5: *Lissachatina fulica* chitosan of 300 mg/200 g BW.

DISCUSSION

Hypercholesterol, associated with LDL oxidation, can cause excess lipid peroxidation, which can increase oxidative stress on lipids under high conditions (Yang et al., 2008). This research shows that animal modeling with four-week hypercholesterol tests increased total cholesterol, triglyceride, LDH level, and decreased HDL level (Table 1). The Wistar rats with hypercholesterolemia were fed egg yolks and fructose orally according to their body weights. It was found that the treatment was able to increase total cholesterol, LDL, and triglyceride levels. Wistar rats have vital cholesterol defense system plasma characteristics and are resistant to hypercholesterol development (Minhajuddin et al., 2005). The lab animal modeling on rats under the hypercholesterolemic condition of

Groups P1, P2, P3, P4, and P5 showed an increase in lipid profile (total cholesterol, triglyceride, and LDL).

The average LDL of Group P0 was 26.8 ± 1.2 mg/dL, which means that the LDL level in rats was normal. Group P1 had an LDL level of 86.7 ± 0.9 mg/dL. The level was three times higher than Group P0. The increase in LDL levels would increase lipid peroxide levels (Minhajudding et al., 2005). Clinically and epidemiologically, the increase in LDL levels can trigger cardiovascular disease (Keevil et al., 2007). In addition to the increase in LDL level, the P2 Group also experienced an increase in total cholesterol and triglyceride level and a decrease in the HDL level. This condition could trigger many diseases.

The mechanism of decreasing cholesterol, triglyceride, and LDL levels using *Lissachatina fulica* chitosan as a natural antioxidant is carried out by preventing lipid peroxidation. Chitosan has an amine group (NH_2) and hydroxyl (OH) as an antioxidant (Mahae et al., 2011; Rajalakshmi et al., 2013; Xie et al., 2001) and hydrogen bond (Xie et al., 2001; 2002). Previous studies stated that chitosan could have a full stomach effect on rats so that it can lower total cholesterol levels and does not affect intestinal cholesterol absorption or fecal sterol output (Van Bennekum et al., 2005). Chitosan can lower total cholesterol levels due to a high-fat diet and lower lipid absorption in absorption organs (Neyrinck et al., 2009; Zhang and Xia, 2015). This research found that chitosan treatment groups, P5 and P4, were more effective than ezetimibe administration (P2), an inhibitor of intestinal cholesterol absorption (Wang et al., 2017).

In addition, *Lissachatina fulica* chitosan can inhibit cholesterol mycelium in the small intestine digestive tract, causing a decrease in total cholesterol, LDL, and triglyceride levels to be absorbed in enterocyte cells. *Lissachatina fulica* chitosan inhibited cholesterol absorption from micelles and the reabsorption of bile acids and synthetic cholesterol. *Lissachatina fulica* chitosan with bile acids formed a great mixture in the micelles, which could not be absorbed by the small intestine and excreted through feces. This research shows that chitosan from snail shells had activities that could reduce total cholesterol, LDL, and triglyceride and increase HDL levels in male Wistar rats with hypercholesterolemia fed with high-fat diets. Chitosan can increase HDL levels due to high-fat diets (Zhang and Xia, 2015).

HDL plays an important role as a cardioprotective by transporting excess cholesterol accumulation in peripheral tissues (macrophages in the aorta). The excess cholesterol is carried to the liver to be excreted into feces through the bile (Wang et al., 2017). Therefore, *Lissachatina fulica* chitosan could inhibit lipid peroxide and lipoprotein oxide under hypercholesterolemic conditions. Fig. 2 below illustrates the mechanism of chitosan in reducing total cholesterol, LDL, and triglyceride.

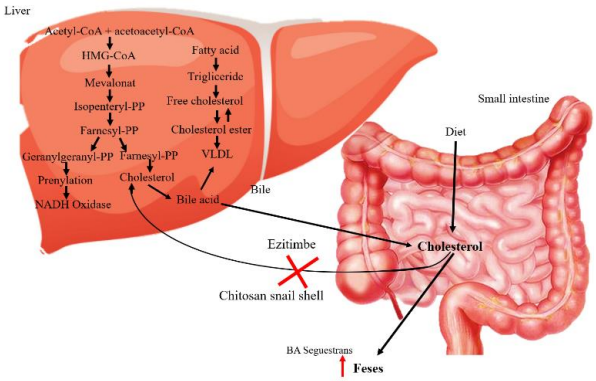

Figure 2. Illustration of a decrease in lipid profile (cholesterol total, LDL, and triglyceride).
Modified from Wang et al. (2017).

The results of the MDA level measurement (Table 5) showed that the highest average MDA level was in Group P1 (10.0 ± 0.2 nmol/mL). The MDA level increased in rats under hypercholesterolemic conditions (Matias et al., 2014; Ngestiningsih et al., 2019; Wahjuni, 2014) can cause oxidative stress (OS) due to the high level of produced reactive oxygen species (ROS) and an increase in lipid peroxidation (Juan et al., 2021; Sachdev et al., 2021; Su et al., 2019). The increased production of ROS can degrade PUFA and form malondialdehyde (MDA), marked by the MDA level in the P1 group that was significantly increased ($p<0.05$) compared to Groups P0 (10.0 ± 0.2 nmol/mL), P2 (2.1 ± 0.3 nmol/mL), P3 (5.6 ± 0.4 nmol/mL), P4 (2.3 ± 0.5 nmol/mL), and P5 (1.2 ± 0.1 nmol/mL). In addition, a high MDA level can indicate increased free radical activity and decreased superoxide dismutase (SOD) enzyme Brunton et al., 2011; Lecumberri et al., 2007; Wahjuni, 2014). A previous study reported that the group of hypercholesterolemic Wistar rats experienced an increase in the MDA level (Fki et al., 2005). Interestingly, this research showed that the higher dosage of *Lissachatina fulica* chitosan administered to Wistar rats for 30 days lowered the MDA level significantly. Therefore, *Lissachatina fulica* chitosan could protect the body from hypercholesterolemia.

Chitosan acted as an antioxidant that could inhibit the lipid peroxidation process by catching free radicals from donating hydrogen atoms, NH_2, and OH, so that more stable compounds were formed. This process also stopped the chained reaction of lipid peroxidation. In this research, the MDA levels of hypercholesterolemic groups were higher than those

given the chitosan treatment of 300 mg/200 g BW, comparable with the MDA level of the control group without being fed a high-fat diet. Because of the antioxidant from the *Lissachatina fulica* chitosan, the researchers could resist the effect of free radicals during the feeding of high-fat diets on Wistar rats through cholesterol absorption in the intestine.

Lissachatina fulica chitosan is an exogenous antioxidant that can protect the body from the free radical reaction so that there is no further lipid peroxidation. It can also lower the formation of MDA levels, proved by the higher the dosage of *Lissachatina fulica* chitosan, the lower the MDA level. A previous study stated that hypercholesterolemic groups have two times higher MDA levels, and oxidative stress with progressive hyper cholesterol is also higher (Yang et al., 2008; Ngestiningsih et al., 2019).

The lowest SOD level indicated by Group P1 was 29.5 ± 2.9 unit/mL. The decrease in SOD level was caused by the role of SOD, which was the first agent in protecting cells from oxidative disorder in the anion superoxide and hydrogen peroxide excretion (Yang et al., 2008). The SOD level decreased in Wistar rats under hypercholesterolemic conditions (Setiawan et al., 2016; Wahjuni, 2014). Meanwhile, SOD levels increased significantly in groups P5, P4, and P3 (41.8 ± 4.3 unit/mL, 63.1 ± 2.1 unit/mL, and 74.6 ± 2.1 unit/mL, respectively) ($p<0.05$). These findings proved that *Lissachatina fulica* chitosan is an exogenous antioxidant that can protect the body from free radical reactions, so that lipid peroxidation does not occur. SOD is an endogenous antioxidant that plays a role in resisting free radicals by changing superoxide ions to stable hydrogen peroxide (Agarwal et al., 2010; Nimse and Pal, 2015).

The increased SOD level in Groups P5 and P4 was possibly caused by the cell defense mechanism due to increased free radical production. Chitosan can increase SOD activity (Zhang and Xia, 2015) and inhibit oxidation reactions, while Group P2, with hypercholesterolemia, showed a decrease in SOD level. It caused peroxiding balance and experienced antioxidant disorders. The possibility of the release of nitrite oxide (NO) and a decrease in the body's micronutrient levels make the cells more sensitive to oxidative stress and lower SOD level (Afonso et al., 2013; Boaventura et al., 2012)

The study showed that there was a correlation between MDA and SOD levels. The higher the MDA level, the lower the SOD level of the Wistar rats, and vice versa. The decrease in MDA level and increase in SOD level occur in hypercholesterolemic Wistar rats after treating germinated brown rice (Matias et al., 2014). Another study showed that the Wistar rats fed high-fat diets have higher MDA levels that can lower SOD levels (Afonso et al., 2013; Matias et al., 2014; Noeman et al., 2011). In this research, the optimal dosage of 300 mg/200 g BW for the Wistar rats proved to be effective in preventing the increase of MDA level and could increase SOD level compared to the control group or other dosages. The previous study also stated that humans' high-fat diets triggered oxidative stress from the higher free radicals and lower endogenous antioxidants (Ngestiningsih et al., 2019; Yang et al., 2008). Therefore, *Lissachatina fulica* chitosan can be a source of antioxidants for health under hypercholesterolemic conditions because it can protect the body from oxidative disorders caused by ROS. It was proved by the lower total cholesterol, triglyceride, LDL level, and significantly higher LDL. Therefore, *Lissachatina fulica* chitosan can reduce peroxide, restore the capacity of antioxidants in the body, and prevent other diseases

CONCLUSION

Lissachatina fulica chitosan is an exogenous antioxidant that can protect the body from free radical reactions. This research showed that male Wistar rats with hypercholesterolemia given *Lissachatina fulica* chitosan exhibited low total cholesterol, LDL, triglyceride, and MDA levels and high HDL and SOD. This research also found that *Lissachatina fulica* chitosan treatment was more effective than the control drug ezetimibe, as an agent to lower cholesterol. The findings prove that *Lissachatina fulica* chitosan can be used to treat and manage hypercholesterolemia.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Umarudin	Widyarti S	Warsito	Rahayu S
Concepts or ideas	x			
Design	x	x	x	x
Definition of intellectual content	x	x	x	x
Literature search	x	x		x
Experimental studies	x			
Data acquisition	x			
Data analysis	x	x	x	x
Statistical analysis	x			
Manuscript preparation	x	x	x	x
Manuscript editing	x	x		x
Manuscript review	x	x	x	x

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