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# The Potential of Green Grape (*Vitis vinifera* L) Extract on Paraoxonase-3 Serum Levels in Rats were Given High Cholesterol Diet

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**Grapes are rich in bioactive molecules that can act as antioxidants, antimicrobials, anti-inflammatory and anti-cancer. Paraoxonase (PON) is an enzyme that can combine with HDL and function as an antioxidant that can protect LDL and HDL from lipid peroxidation which can prevent atherosclerosis. We used a high-cholesterol diet given to Wistar Rats to find out the effect of green grape extract (GGE) on the production of PON3 serum levels in rats. GGE was made using the maceration method. Serum PON3 levels were measured using the ELISA method and measured at 450 nm. The results showed that the highest PON3 serum levels were found in the rat group which was given a GGE dose of 500 mg/200 g BW/day (AH2), followed by levels in rats given GGE at a concentration of 250 mg/200 g BW/day (AH1). Tamhane's test showed that there was a difference between rats that were only given a high-cholesterol diet (DTK) and AH1, AH2, and rats that were fed a standard diet (DS) with AH1 and AH2. However, no difference was found between AH1 and AH2. The higher the dose of GGE given, the higher the serum PON3 level. Further research can better analyze the active compounds in GGE in increasing serum PON3 levels.**

**Keywords:** Atherosclerosis; Coronary heart disease; Green grape extract; Paraoxonase-3.

Coronary Heart Disease (CHD) is a general term used for all disorders involving the obstruction of blood through the coronary arteries which function to supply oxygen to the heart muscle. CHD is the most common cardiovascular disease.[1] Heart and circulatory disease cause around 1 in 3 (estimated 34% in 2019) of deaths globally which means 19 million deaths every year with an average of 50,000 people every day or one death every 1.7 seconds. Globally, heart and circulatory disease are the world's biggest killers. In 2019, CHD is the biggest single killer globally and stroke is the second biggest, both for men and women.[2]

The 2018 Basic Health Research results state that it was found that Non-Communicable Diseases (NCDs) dominate the causes of mortality in Indonesia. CHD is one of the highest NCDs in Indonesia, followed by cancer and diabetes mellitus with complications. The results show that as many

as 1,017,290 people suffer from heart disease based on doctors, diagnoses throughout Indonesia, of which 16,481 people, or 1.3% of them come from the Province of Bali.[3]

CHD is associated with atherosclerosis and dyslipidemia. Dyslipidemia is caused by an imbalance in cholesterol and is characterized by increased levels of cholesterol, low-density lipoprotein (LDL), and/or, triglyceride and decreased levels of high-density lipoprotein (HDL). Cholesterol imbalance and poor immune response will continue with inflammation of the blood vessel walls. Disruption of lipid balance and immune response will result in increased activity of leukocytes, especially monocytes, and homeostasis regulated by chemokines and their receptors. These receptors trigger complex intracellular signaling cascades that stimulate the production of pro-inflammatory cytokines and other inflammatory mediators.[4]

Paraoxonase (PON) is a group of three identified enzymes: paraoxonase-1, paraoxonase-2, and paraoxonase-3.[5] PON can prevent atherosclerosis by forming antioxidant enzymes. Antioxidant enzymes are formed due to the combination of HDL and PON which can prevent lipid peroxidation of HDL and LDL.[6] Two studies reported the contribution of PON3 in a mouse model in vivo. Transient expression of human PON3 using the adenovirus as a vector, was sufficient to suppress the development of atheromatous lesion formation in 26-week-old female apoE null mice compared to mice administered the empty vector, and adenovirus-mediated transgene expression was detectable in rat liver during several studies. Such expression was also detected in the lung, spleen, aorta, and kidney.[7] another study found two human PON3 lines in transgenic mice expressed in highly detectable amounts in several organs including the kidney, liver, and lung. Liver extract isolated from human PON3 transgenic mice showed 56% higher activity in hydrolyzing lovastatin (a PON3-specific substrate) compared to non-transgenic controls. Introduced a human PON3 transgene into LDL receptor knockout mice and demonstrated significantly lower atherosclerotic lesions found in C57BL/6J wild type and C57BL/6J null LDL receptors. This study demonstrated that increased PON3 expression significantly decreased atherosclerosis lesion formation and adiposity in male mice. PON3 may play an important role in protection against obesity and atherosclerosis.[8]

PON prevents the oxidation of arterial cells and lipoproteins, especially cholesterol ester and phospholipid oxidation. PON serum was found to decrease in patients with diabetes mellitus, post-myocardial infarction patients, and patients with familial hypercholesterolemia.[9] PON serum activity depends on several conditions and one of them is food intake. Foods rich in saturated fat can reduce serum PON,[10] whereas those rich in unsaturated fat and moderate consumption can increase serum PON. Low-dose red grapes polyphenolic extract significantly reduced plasma homocysteine levels and restored lever function and plasma PON in mice induced by chronic hyperhomocysteinemia.[11]

Grapes contain many bioactive molecules that are useful for the health of the human body. These compounds such as flavonoids, phenolic acids, lipids, anthocyanins, and stilbenes. Grapes have higher antioxidant activity compared to other plants. Besides being able to function as antioxidants, compounds in grapes also function as anti-inflammatory and anti-cancer. A study state that grapes extracts are rich in bioactive compounds associated with reduced incidence of cardiovascular disease.[12,13]

Bioactive compounds in grapes are reported to provide good efficacy in preventing and treating cardiovascular disease,[13] also an increase in nitric oxide (NO) production, which will improve endothelial dysfunction and hypertension in endothelial cell cultures treated with white grape extract.[14] Proanthocyanidins found in grape seeds suggested can enhance left ventricular remodeling by lowering systolic blood pressure, regulating levels of vasoactive substances, and reducing oxidative stress in spontaneously hypertensive rats.[15]

Previous research conducted showed that the effect of red grapes ethanol extract on reducing triglyceride levels in white rats fed hypercholesterolemia showed a significant reduction in blood cholesterol levels.[16] Subsequent research on green grapes showed the result that the administration of green grape extract had a greater effect on lowering triglyceride levels compared to given simvastatin.[17]

Based on its effect on the lipid profile, researchers were interested in knowing the effect of green grape extract (*Vitis vinifera L*) on paraoxonase-3 serum levels in rats that were given a high-cholesterol diet.

## MATERIAL AND METHODS

The research design used was purely experimental using a pre-test and post-test control group design. The rats used were white rats Wistar (*Rattus norvegicus*), male, weighing 180-200 grams, and aged 3-4 months. Ethical for this research obtained from Polytechnic of Health Denpasar, Bali, Indonesia. with no: LB.02.03/EA/ KEPK/ 0034 /2022.

The research was carried out in several places. The research was conducted at the Animal Laboratory and the Laboratory of Medical Laboratory Technology of the Polytechnic of Health Denpasa. The time of research was carried out from March to October 2022.

The sample size used was calculated using the Federer formula, namely (t-1) (n-1) < 15 so the sample size used was 5 individuals for each treatment group. In this study there were 5 treatment groups namely DS was a group of rats that were only given standard feed, DTK was a group of rats that were given a high cholesterol diet, SS was a group of rats that were given simvastatin at a dose of 0.8 mg/kgBW/day, AH1 was a group of rats which were treated with green grape extract (GGE) at a dose of 250 mg/200 gBW/day, and AH2 was a group of rats that were treated with GGE at a dose of 500 mg/200 gBW/day.

**Preparation of green grape extract (GGE)**

Wash the green grapes (*Vitis vinifera L*) under running water and drain. Cut the green grapes into thin slices, dry the green grape slices in the shade until the green grape slices become dry, and then crush the sun-dried green grapes into powder. The powdered green grapes are then filtered to gain a fine powder. The fine powder is then soaked in ethanol with a concentration of 96% for 24 hours to extract the active ingredient from green grapes. Filtration is done by layering filter paper on a glass funnel. Filtering was carried out to obtain the liquid extract of the grapes, which was then evaporated to a viscous liquid extract using a rotary evaporator.

**The Manufacture of high-cholesterol feed**

The high cholesterol feed given to experimental rats consisted of 50% standard feed, 31.8% of flour, 1% of cholesterol, 0,2% of cholic acid, 10% of lard oil, 2% of pig brain, and 5% of egg yolk. All the ingredients are mixed and ground and then formed into small granules and dried.

**Preparation of simvastatin solution**

Prepare simvastatin solution by dissolving 10 mg of simvastatin in 100 ml of distilled water so that 1 ml of solution contains 0.1 mg of simvastatin. The dose used in rats with an average body weight of 200 g was 0.2 mg/day.

**Rats blood collection**

Blood was taken at the end of the 4$^{th}$ week of treatment. The rats were anesthetized and then blood was taken from the orbital sinuses for serum PON3 examination using the ELISA method.

**The Examination of PON3 levels**

The rat blood was allowed to coagulate for two hours at room temperature before being centrifuged for 20 minutes at 1000 rpm. Samples were stored at -20$^0$C. The standard solution was diluted to various concentrations. 100 ìl each i.e., standard solution, negative control, and samples were put into the well. Then closed using a sealer. Incubated for 2 hours at 37$^0$C. The discarded solution added 100 ìl of detection reagent A and incubated for 1 hour at 37$^0$C. Cover again with sealer. Discarded the solution and washed with 350 ìl 1X washing buffer for each well then incubated for 2 minutes. Discarded the solution, and washed it 3 times. Dry well with absorbent paper. 100 ìl of detection reagent B was added, covered with a sealer, and then incubated for 1 hour at 37$^0$C. The solution was discarded and washed again 5 times. 90 ìl of substrate solution was added and then covered with a sealer. Incubated for 20 minutes at 37$^0$C and protected from the light. The liquid will turn blue with the addition of the substrate solution. 50 ìl of stop solution was added and the liquid will turn yellow. Serum PON3 levels were measured on a microplate reader at 450 nm.

**Data analysis**

A descriptive analysis of research data was carried out to obtain the average serum PON3 levels in the control and treatment groups.

Analysis of normality and homogeneity of data. This analysis serves to determine the

distribution of data on serum PON3 levels. The normality of the data in this study was tested using the Saphiro-Wilk Test. To find out the homogeneity of the data, the Lavene Test was carried out.

Comparative analysis. This test was conducted to compare the average post-test data. If the data is normally distributed, it will be tested with the One-Way ANOVA test. If it is significant, it will be tested with Kruskal Wallis. Data analysis using computer assistance using a 95% confidence level.

## RESULTS AND DISCUSSION

The results obtained in this study were an analysis of paraoxonase-3 (PON 3) levels of rat serum after being treated with green grape extract (*Vitis Vinifera L*). The results of this stage will be presented as follows:

After being treated for 4 weeks, the rats were taken for blood to check the serum PON3 levels. PON3 serum levels obtained can be seen in Table 2.

Table 2 shows that the highest average serum PON3 levels were found in the AH2 group, which was the group given GGE at a dose of 500 mg/200 gBW/day, while the lowest average levels were found in the rat group that was given only high cholesterol feed.

The results of the homogeneity test showed that the data was not homogenous and all data were normally distributed. The results of the normality test using the Shapiro-Wilk test and homogeneity using the Levene test are presented in Table 3.

Based on the results of the normality test, then the difference test was then carried out using One-Way ANOVA. The test results using the One-Way ANOVA. This test showed that there was a significant difference between the treatment groups with a $p$=0.000. Then proceeding with the Tamhane test showed differences were found to a large extent between the groups. Whereas no difference was found between the DS and SS groups and the AH1 and AH2 groups. The test results are shown in Table 4.

Table 4 shows that there is an effect of giving GGE on the PON3 levels of rat serum. This is evidenced by the difference between the DS - DTK, DS - AH1, and DS - AH2 groups. As well as between the DTK - SS, DTK - AH1, and DTK - AH2 groups. Whereas in the AH1 and AH2 treatment groups, there was no difference.

**Table 1.** The Average Body Weight

| Groups | Average Body Weight of Rat (gram) | | |
|---|---|---|---|
| | Early Week | Before Treatment | After Treatment |
| DS | 204.6 | 209.8 | 188.4 |
| DTK | 219.6 | 212.6 | 213.6 |
| SS | 260.2 | 226.2 | 233.2 |
| AH1 | 239 | 215.6 | 222.4 |
| AH2 | 239.6 | 242.8 | 268.2 |

**Table 2.** The Average of Paraoxonase-3 Serum Levels

| Groups | Rats Serum PON3 Levels (ng/ml) | |
|---|---|---|
| | Mean | Standard Deviation |
| DS | 5.652 | 0.436 |
| DTK | 2.288 | 0.373 |
| SS | 5.401 | 0.420 |
| AH1 | 8.519 | 0.093 |
| AH2 | 12.622 | 0.933 |

**Table 3.** The Normality and Homogeneity Test Results for Paraoxonase-3 Levels

| Groups | Test Results | |
|---|---|---|
| | Homogeneity | Normality |
| DS | 0.007 | 0.677 |
| DTK | | 0.902 |
| SS | | 0.345 |
| AH1 | | 0.340 |
| AH2 | | 0.756 |

Meanwhile, based on Graph 1, it can be seen that the concentration of PON3 levels increased when given a larger dose of GGE.

The difference in PON3 levels between the study groups showed that the administration of GGE had a different effect compared to the administration of no extract or the administration of simvastatin. The group that was given standard feed had almost the same mean PON3 levels and showed no difference from the group that was given simvastatin. This showed that simvastatin therapy has the effect of increasing PON3 levels in rats that have been given a high-cholesterol diet, with PON3 levels approaching the PON3 levels in the serum of rats that consume the standard feed.

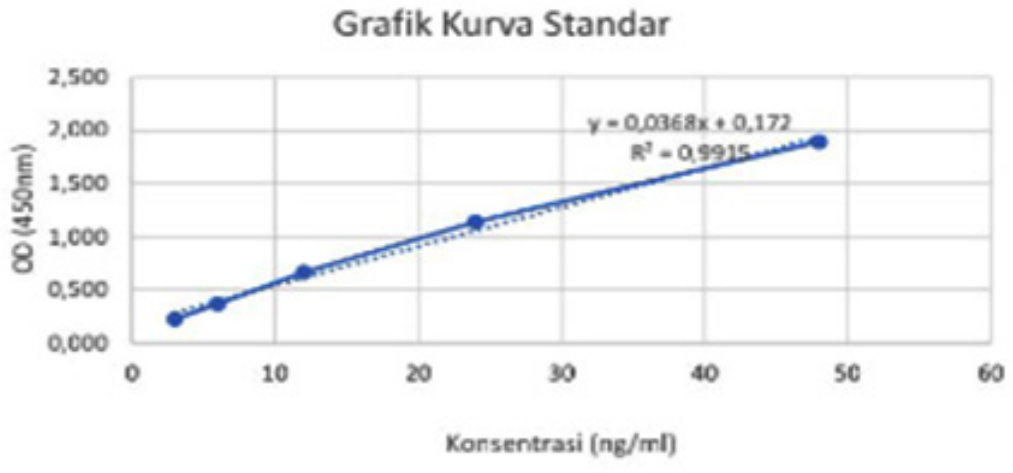
PON3 levels showed differences in the standard feed group, high-cholesterol diet, GGE treatment at a dose of 250 mg/200 gBW/day, and GGE at a dose of 500 mg/200 gBW/day. Administration of GGE at a dose of 500 mg/200 gBW/day had the greatest effect in increasing rat serum PON3 levels. It was found that the higher the dose given to the samples, the more active ingredients the samples absorbed, namely phytosterol, anthocyanins, resveratrol, and tannins. In doing so, it demonstrated the ability to further increase PON3 levels.

**Table 4.** The Difference Test Results for Paraoxonase-3 Levels

| Groups | Tamhane Test Results |
|---|---|
| DS-DTK | 0.004 |
| DS-SS | 0.021 |
| DS-AH1 | 0.019 |
| DS-AH2 | 0.011 |
| DTK-SS | 0.000 |
| DTK-AH1 | 0.005 |
| DTK-AH2 | 0.011 |
| SS-AH1 | 0.039 |
| SS-AH2 | 0.018 |

The results showed that there was an effect of giving GGE on rat serum PON3 levels. This is related to the ability of GGE to improve lipid profiles, one of which is to increase levels of HDL.[18] GGE can increase blood HDL due to the presence of phytosterols which compete with cholesterol for absorption in the intestine, causing more cholesterol from food to be stored in the liver so that cholesterol levels in the blood decrease. In addition, the reduction of cholesterol levels is also due to the combination of tannic acid and bile acid in the intestine, which is excreted with the feces, thereby affecting the reduction of cholesterol levels. GGE is rich in phenolics and flavonoids, with polyphenols increasing plasma HDL levels.[19]

Over the past few years, more and more research has been conducted on the polyphenol content in grapes due to their potential to reduce cardiovascular disease. In addition to the fruit, the skin, pulp, and seeds also contain flavonoids, resveratrol, and phenolic acids in varying levels, depending on the grape species and geographic origin.[20,21]




**Graph 1.** Graph of Average Standard Curve of PON3 Levels

PON3 is a calcium-dependent glycoprotein. These glycoproteins have a total weight of 40kDa. PON3 functions to catalyze the hydrolysis of many substrates, one of which is a pharmacological agent.[22] Most of PON3 is expressed in the liver and plasma bound to HDL.[6] PON3 is known to act as an antioxidant, suggested that PON3 was found to protect LDL against copper-induced oxidation in vitro.[23]

PON3 in circulation can be associated with HDL and is also found in atherosclerosis plaques.[24] On apolipoprotein profile analysis by High-Performance Liquid Chromatography (HPLC) showed that PON3 expression was present between fractions 28 and 31, along with PON1, on apoA-I containing particles, but not in apoA-II or apoE.[25] PON3 is reduced from HDL in patients with subclinical atherosclerosis, indicating that PON3 is an important antioxidant protein in preventing atherosclerosis.[26] With the discovery of PON3 in particles containing apoA-I from HDL, an increase in HDL will be followed by an increase in apoA-I so that PON3 also increases.[27]

In this study, there are still shortcomings, namely the lack of the best dose variation to produce the optimal dose to increase PON3 levels. Besides that, it is also necessary to study the variation of doses by administering the extract over a longer period to find out the toxic dose, side effects, and the most effective duration of extract administration.

## CONCLUSION

The conclusion of this study is: there were differences in the levels of PON3 in each study group, namely at the standard feed, high-cholesterol feed, simvastatin, GGE at a dose of 250 mg/200 gBW/day, and GGE at a dose of 500 mg/200 gBW/day; GGE (*Vitis vinifera L*) has the effect of increasing PON3 levels; GGE with a dose has a greater effect on increasing PON3 levels compared to the positive control given simvastatin; and GGE at a dose of 500 mg/200 gBW/day had a greater effect on increasing PON3 levels on average, but in the treatment group there was no difference between treatment doses.

For further research, it is better to do analytical research on the active compounds found in green grapes in increasing serum PON3 levels. Then carried out further research on the dosage of the extract was made more varied with due regard to the maximum dose of the extract and the effects of a grape extract on humans. As well as follow-up research of the same type using a larger sample size.

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None.

**Conflict of Interest**

The authors have no conflict of interest to declare. All co-authors have seen and agree with the contents of the manuscript. We certify that the submission is original work and is not under review at any other publication.

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