The Potential of Black Cumin SNEDDS (Self-nanoemulsifying Drug Delivery System) on Total Cholesterol Level of Hypercholesterolemic Model Zebrafish (Danio Rerio)

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Abstract. Hypercholesterolemia is a risk factor for various cardiovascular diseases. This condition is marked by the increasing total cholesterol level higher than 200 mg/dL. Black cumin is one of the herbal plants which has the potential to be an antihyperlipidemic agent. Black cumin used in this research is SNEDDS-based, which can increase drug bioavailability. This study aims to determine the effect of SNEDDS-based Black Cumin on Zebrafish total cholesterol levels. This is quasi-experimental research with pre-test and post-test nonequivalent control group design. The research subjects were divided into four groups: a positive control group (Simvastatin 20 mcg/g feed), a negative control group (without intervention), Intervention 1/P1 (25 ppm SNEDDS), and Intervention 2/P2 (50 ppm SNEDDS). The obtained data were analyzed with Shapiro-Wilk Test, Kruskal-Wallis Test, and Wilcoxon Test. Total cholesterol level decreased significantly in the P1 group, with the total cholesterol pre and post-test difference up to 100.4 $\pm$ 81.89 and p-value 0.043 ($p < 0.05$). Otherwise, in the P2 group, the positive control group, and the negative control group did not show any significant differences between pre and post-test total cholesterol levels with the p-value 0.345 ($p > 0.05$), 0.5 ($p > 0.05$), and 0.686 ($p > 0.05$) respectively. Black Cumin SNEDDS, which has a concentration of 25 ppm, can decrease total cholesterol levels on hypercholesterolemic zebrafish.

Keywords: Hypercholesterolemia · zebrafish · black cumin seeds oil · SNEDDS

1 Introduction

Hypercholesterolemia is a condition characterized by a very high plasma cholesterol level, with a total cholesterol level above 200 mg/dL. This increase in blood cholesterol levels disrupts and changes the structure of blood vessels, causing endothelial function disorders to form lesions, plaques, occlusions, and even emboli [1]. These endothelial

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changes underlie the occurrence of various diseases, such as atherosclerosis which leads to stroke. This condition is quite hard to detect, and the only way to determine cholesterol levels in the blood is to do a lipid profile test [2].

Hypercholesterolemia is a health problem that happens commonly. Globally, one-third of ischemic heart disease is given rise to high cholesterol levels, and this condition is estimated to cause 2.6 million deaths. This increase in total cholesterol is a crucial cause of disease burden in developed countries and is a developing risk factor for heart disease and ischemic stroke [1].

Europe has the highest prevalence of increased total cholesterol at around 54%, followed by America at around 48%, then Africa and Southeast Asia that have the lowest percentage at around 22.6%. These results indicate that a high prevalence of hypercholesterolemia is influenced by the welfare level of the country's population. However, even though Indonesia is one of the countries with the lowest prevalence of hypercholesterolemia, the habit of consuming fatty food is difficult to avoid. On the other hand, Indonesia is recorded to have a percentage of obesity of 21.8% and the prevalence of central obesity at the age of 18 years old from 2007–2018 continues to increase, from 18.8% to 31.0% [3].

Black cumin or Black Seed is one of the herbal plants that have various benefits for the body, one of which is as an antihyperlipidemic agent. Black Seed has been proven to reduce LDL levels, a lipid substance that causes atherosclerosis [4].

The Self-Nanoemulsifying Drug Delivery System (SNEDDS) is currently being developed to improve the quality of drug performance, especially in its pharmacokinetic processes. SNEDDS is a further processed form of the extract, so it is supposed to have a better effect than the extract form. This mechanism is because SNEDDS has a nano-size that allows it to be absorbed better by the body so that its bioavailability is greater. Although its size is tiny compared to other forms of drug delivery system, SNEDDS has been tested for its physical and chemical stability [5]. This study aims to determine the effect of SNEDDS-based Black Cumin on Zebrafish total cholesterol levels.

2 Method

This research is a quasi-experimental study with pre and post-test nonequivalent control group design conducted at the Animal Laboratory of the Faculty of Mathematics and Natural Sciences and the Research Laboratory of the Faculty of Medicine, Universitas Islam Indonesia (UII) from November 2020 until February 2021. This research has received approval from the Medical Ethics and Health Research Committee, Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada with protocol number: KE/FK/1123/EC/2020.

The inclusion criteria of the subjects in this study were: zebrafish aged 4–6 months, male and female, and healthy as indicated by an active swimming pattern. Besides, the exclusion criteria for the subject of this study were sick and dead zebrafish that were not because of the intervention.

The study began by acclimatizing zebrafish for seven days. Several things need to be considered in the care of zebrafish during this research, including the availability of an aquarium circulation system consisting of canister filters, aerators, and oxygen. The

aeration system was supplied by using a pump and a pipe connected to an oxygen hose into each aquarium. In addition, the lighting duration in the laboratory should be adjusted with a pattern of 14 h of light and 10 h of darkness. The e temperature was maintained in the range of 26–28.5 °C and a pH of around 6.8–7.5 to minimize the presence of confounding factors during the study. This condition must be checked twice a day, at 9 am and at 7 pm. The aquarium was drained once a week to keep it clean [6].

After acclimatization for seven days, zebrafish were divided into four groups in four different aquariums. The four groups used in this study consisted of a 25 ppm black cumin SNEDDS treatment group (P1), a 50 ppm black cumin SNEDDS treatment group (P2), a group that was given a mixture of feed and Simvastatin as much as 20 mcg/g of feed (positive control), and the group that was only given induction but no intervention (negative control). After acclimatization for seven days, zebrafish were divided into four different aquariums with the group division mentioned above. Afterwards, the induction process was carried out for 14 days with an induction feed made from a mixture of standard Hikari feed and boiled broiler egg yolks with a ratio of 0.49 g of feed: 0.1 g of egg yolk in all groups [7].

After the induction process was completed, then zebrafish blood was drawn using the decapitation technique to measure total cholesterol levels after induction. Zebrafish cholesterol levels were measured using the LipidPro strip test kit.

After obtaining data on measuring total cholesterol levels post-induction, the study was continued by giving treatment according to group division for 14 days. Furthermore, zebrafish blood was taken using the decapitation technique to obtain data on total cholesterol levels after the intervention.

3 Result and Discussion

The subjects used in this study were adult zebrafish (Danio rerio) aged 4–6 months with inclusion criteria; healthy fish indicated by fish that appeared to be actively moving. On the other hand, the subject exclusion category in this study was whether the fish looked sick or died during the acclimatization and treatment period. This study used 14 zebrafish per aquarium and was conducted with four groups consisting of two treatment groups and two control groups.

Zebrafish were acclimatized for seven days so that the fish could adapt to laboratory conditions. After acclimatization, zebrafish were ready to be treated. The first treatment was performed by providing a high-fat induction feed consisting of fish feed mixed with egg yolk. High-fat induction was administered for 14 days, after which decapitation was performed to draw blood and check total cholesterol levels.

The results of measuring total cholesterol levels after the induction of hypercholesterolemia are as follows (Table 1).

The condition of hypercholesterolemia is characterized by a total cholesterol level of more than equal to 200 mg/dL. The results above showed an increase in total cholesterol levels in all treatment groups after induction feeding with egg yolk.

Treatments were applied to 4 groups of fish consisting of a treatment group that was given SNEDDS Black Cumin with a concentration of 25 ppm (P1), SNEDDS Black Cumin with a concentration of 50 ppm (P2), a positive control group (K+), and a negative

Table 1. Results of measuring zebrafish total cholesterol levels after hypercholesterolemia induction.

Tests	Total Cholesterol Levels			
	P1 (mg/dL)	P2 (mg/dL)	K + (mg/dL)	K− (mg/dL)
Pre-test	275.8 ± 92.36	205.4 ± 101.82	237.8 ± 109.5	224.8 ± 113.48
Post-test	175.4 ± 94.99	168.4 ± 46.56	223 ± 105.1	235.6 ± 77.19

* P1 = 25 ppm black cumin SNEDDS treatment group, P2 = 50 ppm black cumin SNEDDS treatment group, K+ = positive control group, K− = negative control group.

control group (K−). The positive control group was given standard fish feed mixed with Simvastatin in a ratio of 20 mcg/g of feed. After 14 days of treatment, zebrafish blood was collected by the decapitation method. The results of measuring total cholesterol levels after the intervention are as follows.

The results above found that the group treated with the black cumin SNEDDS experienced a decrease in total cholesterol levels until they reached normal levels, which were below 200 mg/dL. Meanwhile, total cholesterol levels decreased in the positive control group but did not reach normal levels and were still above 200 mg/dL. In the negative control group, there was no decrease in total cholesterol levels.

Before analysis, the data distribution normality was assessed using the Shapiro-Wilk test because the amount of data was less than 50. The obtained normality test was $p < 0.05$ in the group that received SNEDDS treatment with a concentration of 25 ppm, while $p > 0.05$ was obtained in the group that received SNEDDS treatment with a concentration of 50 ppm, the positive control group, and the negative control group. Therefore, it concludes that the distribution of the data was not normal.

Furthermore, a multivariate statistical analysis test was conducted to determine whether there was a significant difference between the total cholesterol levels of one group and another. In addition to the multivariate test, bivariate statistical tests were also conducted to assess if there was a significant difference between total cholesterol levels before and after the intervention of each group. The results of the normality test found that the data distribution was not normal. Therefore, the parametric test with One Way ANOVA could not be performed. Instead, a non-parametric test can be performed using the Kruskal-Wallis method to compare the results of total cholesterol levels from one group to another. As for the comparison of total cholesterol levels before and after treatment, the researchers used the Wilcoxon test as an alternative to the Paired T-test.

From the results of multivariate analysis using Kruskal-Wallis in Table 2, the p-value of 0.383 ($p > 0.05$) means that there is no significant difference in total cholesterol levels between groups. It means that the administration of black cumin SNEDDS at a dose of 25 ppm and 50 ppm did not provide a significant difference, as well as when compared to the positive control group and the negative control group. In addition, a bivariate test was performed, and the results are presented in Table 3. Of the four groups, the 25 ppm SNEDDS treatment group was the treatment group that gave significant results on changes in total cholesterol levels before and after treatment indicated by a p-value

Table 2. The results of the Kruskal-Wallis test on the measurement of total cholesterol levels before and after treatment.

Tests	Total Cholesterol levels				p-value
	P1 (mg/dL)	P2 (mg/dL)	K+ (mg/dL)	K− (mg/dL)	
Pre-test	275,8 ± 3.00	205,4 ± 3.00	237.8 ± 3.00	224.8 ± 3.00	0.371
Post-test	175.4 ± 3.00	168.4 ± 3.00	223 ± 3.00	235.6 ± 3.00	0.383

Table 3. Wilcoxon test results to assess the significance of changes in total cholesterol levels before and after treatment

Group	Pre-Intervention Cholesterol Levels (mg/dL)	Post-Intervention Cholesterol Levels (mg/dL)	Pre and Post-Intervention Cholesterol Levels Differences (mg/dL)	*p-value*
P1	275,8 ± 92.36	175,4 ± 94.99	100,4 ± 81.89	0.043
P2	205,4 ± 101.82	168.4 ± 46.56	37.00 ± 87.61	0.345
K+	237.8 ± 109.5	223 ± 105.16	14.8 ± 135.60	0.5
K−	224.8 ± 113.48	235.6 ± 77.19	-10.8 ± 123.41	0.686

of 0.046 ($p < 0.05$). Thus, it concludes that the administration of 25 ppm of black cumin SNEDDS effectively reduces the total cholesterol level of zebrafish.

The obtained p-value of multivariate analysis using Kruskal-Wallis was 0.371 ($p > 0.05$) for total cholesterol levels before treatment and the p-value of 0.383 ($p > 0.05$) for measurement results after treatment, which means there is no significant difference in total cholesterol levels between groups. This result indicates that the administration of black cumin SNEDDS at a dose of 25 ppm and 50 ppm did not provide a significant difference, as well as when compared to the positive control group and the negative control group.

The difference in total cholesterol levels before and after treatment was analyzed for significance by the Wilcoxon test. The interpretation of the Wilcoxon test is to look at the results of the p-value. The difference in total cholesterol levels before and after treatment is significant if the p-value is less than 0.05. Of the four groups above, only the 25 ppm SNEDDS treatment group had a p-value below 0.05, while the other three groups had p-values above 0.05. This condition means that there is a significant difference between total cholesterol levels before and after treatment in the group given 25 ppm of SNEDDS, while in the group given 55 ppm of SNEDDS, the positive and the negative control group did not show a significant difference between the results of total cholesterol levels before and after treatment. This condition means that giving 25 ppm of black cumin SNEDDS is effective to reduce total cholesterol levels in zebrafish.

4 Discussion

This study determines the effectiveness of giving SNEDDS based black cumin to decrease the total cholesterol level of zebrafish hyperlipidemia induced zebrafish. After seven days of acclimatization, zebrafish were induced to become hypercholesterolemia (cholesterol level > 200 mg/dL). This method was by Andriana's research in 2014 and Amanah in 2020 that induction using a mixture of standard feed and egg yolk showed an increase in cholesterol levels. Andriana's study (2014) found that an average increase in cholesterol levels was up to 272 mg/dL, Amanah (2020) found that an average increase in total cholesterol levels was up to 237.4 mg/dL with the same induction [7, 8].

The treatment was performed after the zebrafish had experienced hypercholesterolemia in the four groups. The treatment results showed a decrease in total cholesterol levels in the group given 25 ppm of black cumin SNEDDS, whereas in the group given black cumin SNEDDS at a dose of 50 ppm and the positive control group did not experience a decrease in total cholesterol levels. In addition, the negative control group experienced an increase in total cholesterol levels as shown in the table. From the results of measuring the total cholesterol level of zebrafish, it was found that the 25 ppm of SNEDDS treatment group was the group that showed the highest difference in the total cholesterol levels pre- and post-intervention among the other groups, which was 100.4 mg/dL. The zebrafish group given 50 ppm of SNEDDS black cumin decreased total cholesterol levels by 37 mg/dL, and the zebrafish group given Simvastatin experienced a decrease in total cholesterol levels by 14.8 mg/dL. Nevertheless, the reduction in total cholesterol levels in the group given 50 ppm of black cumin SNEDDS and the group given Simvastatin 20 mcg/g feed did not show any statistical significance. On the contrary, the zebrafish group given 50 ppm of black cumin SNEDDS did not show any decrease in total cholesterol levels compared with the group treated with 25 ppm of black cumin SNEDDS. This study result does not meet the hypothesis that the decrease in total cholesterol levels is directly proportional to the size of the treatment dose. The researchers assume that this happens because of bias in the study. Possibly this is caused by a lack of optimal formulation of SNEDDS. Thus, the substance clumps and does not mix well in the aquarium water. It then resulted in a concentration of 50 ppm not being reached in one aquarium. The clumps of SNEDDS in aquarium water cause the drug's effectiveness to decrease because of the reduction in the dissolution rate of the active substance [9].

Meanwhile, the positive control group also did not experience a significant decrease in total cholesterol levels. The researchers assumed that this might be because the standard drug Simvastatin was not made in the SNEDDS formula, so the comparison between the treatment and control groups was not optimal. The SNEDDS formula itself is known to increase the bioavailability of a drug by increasing its solubility, and Simvastatin used in this study was not SNEDDS based. This condition may cause total cholesterol levels to decrease insignificantly in the positive control group.

There was a significant decrease in total cholesterol levels before and after treatment with 25 ppm of black cumin SNEDDS, as stated by research conducted by Wijaya et al. (2018) in hypercholesterolemic model mice [10]. This study concluded a significant decrease in total cholesterol levels in hypercholesterolemic mice after being given black cumin seed extract for ten days at a dose of 0.168 g/day. Al-Naqeep et al. (2010) stated

that there was also a significant decrease in total cholesterol levels in rabbits induced by hypercholesterolemia after being given black cumin extract based on oil and powder at a dose of 500 mg/kg body weight and 1000 mg/kg body weight in the second, fourth, sixth, and eighth weeks, respectively. In addition to lowering rabbits' total cholesterol levels, black cumin seed extract based on powder and oil increased rabbits' HDL levels. Decreased total cholesterol levels and increased HDL levels of rabbits with black cumin extract showed significant results compared to the positive control group with Simvastatin [11].

The decrease in total cholesterol levels of zebrafish in this study occurred because of the content of black cumin, which can reduce total cholesterol levels. The black cumin extract in this study is SNEDDS based to effectively increase the bioavailability of thymoquinone, the active substance in black cumin which acts as an anti-cholesterol agent.

This study has several limitations, including the obstacles in the acclimatization phase. During acclimatization, too large standard fish feed and bad aquarium hygiene raised the mortality rate in zebrafish up to more than 10% Thus the acclimatization was repeated up to three times. In addition, very little blood came out during the decapitation process. Besides, there is a sampling bias because decapitation causes the blood specimen to not be collected from the same subject between pre-test and post-test. Moreover, the uncertain level of zebrafish satiation is also a confounding variable that was not controlled in this study, and it can cause bias in the results of measuring zebrafish total cholesterol levels. Simvastatin preparations used for treatment in the positive control group also have not been made in the form of SNEDDS, so the comparison with the black cumin SNEDDS treatment group is not optimal.

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