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The Effect of Habbatussauda (*Nigella sativa*) on TNF-α and IL-6 Levels in Wistar Rats Induced High Fat Diet

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ABSTRACT

Habbatussauda (*Nigella sativa*) contains the active substance thymoquinone which has antihypercholesterolemic, antioxidant, and anti-inflammatory effects. Administration of Habbatussauda oil can improve TNF-α and IL-6 levels in hypercholesterolemia conditions. The research aimed to determine the effect of giving habbatussauda on levels of TNF-α and IL-6 in rat fed a high-fat diet. Post-test-only control group design experimental research for 45 days. A sample of 30 Wistar rats aged 8 weeks, weighing 150-200 grams and adapted for 7 days, were randomly divided into 5 groups, namely: normal control group (K1), negative control group given a high-fat diet (K2), treatment group given habbatussauda 0.001 mL/gBB (K3), treatment group given habbatussauda 0.002 mL/gBB (K4), treatment group given habbatussauda 0.004 mL/gBB (K5) treatment was given for the last 2 weeks. TNF-α and IL-6 levels were measured by the ELISA method. Data were analyzed using One Way Anova then continued with the Post Hoc LSD. The mean ± SD of TNF-α levels in groups K1, K2, K3, K4 and K5 were 83.36 ± 23.73, 246.47 ± 43.21, 155.83 ± 36.38, 171.80 ± 22.50, and 195.77 ± 47.67 pg/mL, respectively. In IL-6 groups K1, K2, K3, K4 and K5 were 33.83 ± 8.55, 115.11 ± 16.51, 58.85 ± 19.71, 76.70 ± 6.03, and 78.05 ± 17.33 pg/mL, respectively. One Way Anova test for TNF-α and IL-6 levels showed significant differences between groups (p = 0.000 and p = 0.000). In the post hoc test, TNF-α and IL-6 levels in K3, K4, and K5 were significantly lower than in K2 (p = 0.000). Administration of Habbatussauda can reduce TNF-α and IL-6 levels in rat induced by a high-fat diet.

Keywords: Habbatussauda, *Nigella sativa*, Hypercholesterolemia, Obesity, TNF-α, IL-6

INTRODUCTION

Hypercholesterolemia is a condition in which the concentration of cholesterol in the blood increases beyond normal limits. Hypercholesterolemia can increase systemic pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β which play a role in the formation of atherosclerotic plaque. This condition will cause the development of cardiovascular disease complications (Mohammadi et al., 2017). Statin drugs are often used in the treatment of hypercholesterolemia, however, it is considered unsatisfactory because of the side effects (Erwinanto et al., 2017)،(Namazi et al., 2018). Alternative therapies using natural ingredients are currently being developed, such as Habbatussauda which has anti-hypercholesterolemia, anti-inflammatory and antioxidant effects (Namazi et al., 2018)،(Susilowati et al., 2019)،(Pei et al., 2020). However, there is currently little data regarding studies of the effects of Habbatussauda on TNF-α and IL-6 levels in hypercholesterolemic rat.

The proportion of the Indonesian population in 2018 aged ≥ 15 years who had abnormal lipid levels in the form of total cholesterol was 21.2%, LDL 37.3%, triglycerides 27.9%, and HDL 24.3% (Dany et al., 2020). Hypercholesterolemia is closely related to an increased risk of several chronic diseases, namely type 2 diabetes, atherosclerosis, cardiovascular disease (CVD), non-alcoholic fatty liver disease and several types of cancer (Namazi et al., 2018). The prevalence of heart disease at age ≥ 15 was 18.4%. Complications that arise due to hypercholesterolemia cause dependence to carry out daily activities, especially in old age. The proportion of dependency level at age ≥ 60 years according to disease, namely heart disease 36%, DM 36.41%, and stroke 63.66% (Nugroho et al., 2022).

The results of research on a hyperlipidemic mouse model given a high-fat diet (0.5% cholesterol, 3% coconut oil, and 0.25% cholic acid) for 30 days can cause an increase in pro-inflammatory cytokine levels TNF-α and IL-6 levels were significant (Kumar et al., 2020). The main active compound of Habbatussauda is thymoquinone, which has anti-hypercholesterolemia, anti-inflammatory, and antioxidant properties (Hadi et al., 2021) Habbatussauda has previously been studied in rat given a high-fat diet for 28 days followed by administration of Habbatussauda for 14 days at a therapeutic dose of 450 mg/kg BW, which can reduce MDA levels by 30.20% and show improvement in the histopathological picture of the jejunal mucosa of hypercholesterolemic rat (Lestari,

2018). Other studies also support that administering Habbatussauda powder at a dose of 300 mg/kg/day for 6 weeks after obesity induction can significantly normalize lipid profiles, reduce body weight, serum atherogenic index (AI) and serum ALT (Esmail et al., 2021).

Habbatussauda have agonistic effect on peroxisome proliferator-activated receptor gamma (PPAR-γ) and increasing regulation of LDL receptors in hepatocytes resulting in cholesterol removal, and suppression of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA R) gene which affects cholesterol metabolism, resulting in a reduction in oxidative stress, TNF-α and IL-6. Habbatussauda also works by inhibiting the nuclear factor-kappa B (NF-κB) pathway which can cause increased expression of various types of inflammatory cytokines (IL-1β, IL-6, TNF-α, IFN-γ, and MMP3), COX-2, and iNOS (Hadi et al., 2021).

Research on habbatussauda in influencing cholesterol levels and its ability to control inflammatory processes has been widely carried out, but the lack of studies regarding the effect of habbatussauda on pro-inflammatory cytokines induced by hypercholesterolemia conditions causes the need for further research. Based on this, research is needed to see the effect of Habbatussauda in preventing the inflammatory process that occurs in hypercholesterolemia conditions which can be observed through the levels of TNF-α and IL-6 in male white Wistar rats fed a high-fat diet.

METHODS

This research is an experimental laboratory research with a Post Test Only Control Group Design. Ethical clearance for the research was obtained from the ethics committee of the Faculty of Medicine, Sultan Agung Islamic University, Semarang. The population used in this research was white male Wistar rats. The research sample was taken from the population randomly and met the inclusion, exclusion, and dropout criteria. Inclusion criteria: Male Wistar strain rats aged 6 - 8 weeks, body weight 150-200 grams, and healthy (rats actively move around). Exclusion criteria: anatomical defects in rats. Drop Out Criteria: rat died during the study.

All samples (30 individuals) were divided into 5 groups, with K1: Healthy group, K2: Negative control group: a group of rat induced by a high-fat diet, K3: Treatment group 1: a group of rat induced by a high-fat diet and given 0.001 mL of Habbatussauda / g BW/ day for 14 days, K4: Treatment group 2: a group of rat induced by a high-fat diet and given habbatussauda 0.002 mL/ g BW/ day for 14 days, K5: Treatment group 3: a group of rat induced by a high-fat diet and given habbatussauda 0.004/g BW/day for 14 days.

The dosage of habbatussauda oil used in this study was 2 mL/kg BW or equivalent to 0.002 mL/g BW/day. Based on the half dose and double dose methods, 3 doses of habbatussauda oil were obtained, namely 1 mL/kg (0.001 mL/g BW/day), 2 mL/kg (0.002 mL/g BW/day), and 4 mL/kg (0.004 mL /g BW/day) (Alkadri *et al.*, 2019)،(Al-Gayyar *et al.*, 2016)،(Pop *et al.*, 2020). Habbatussauda oil was administered on days 32-44 of the study or for 14 days by oral sondase (Fabiana Meijon Fadul, 2019). The Habbatussauda used is the Habbasyifa Oil brand with 100% purity which has manufacturing standards and is registered with BPOM with POM number TR 113 323 461 and MUI halal certificate No.00140016360701. One bottle contains 250 mL of habbatussauda oil. The dosage of habbatussauda oil in this study is 1 mL/kg (0.001 mL/gBW/day), 2 mL/kg (0.002 mL/gBW/day), and 4 mL/kg (0.004 mL/gBW/day). Habbatussauda oil was administered on days 32-45 of the study or for 14 days using oral sondage. On day 45, TNF-α and IL-6 levels were checked with the ELISA method

Data were collected and presented descriptively in the form of median and standard deviation, then a data normality test was carried out using the Shapiro–Wilk Test and a homogeneity test using the Levene Test. Data that is normally distributed and has homogeneous variance is analyzed using the one-way ANOVA test and if it is found to be significant, it is continued with the LSD post hoc test. If $p < 0.05$ is obtained then there is a difference between groups.

RESULTS AND DISCUSSION

Based on the results of the normality analysis of data distribution tested by Shapiro Wilk, it was found that each group had a normal data distribution, indicated by a p-value> 0.05 for the TNF-α and IL-6 variables. The results of the analysis of homogeneity of variance tested using the Levene test also showed homogeneous results, indicated by a p-value> 0.05 for the variable. TNF-α and IL-6 were p=0.409 and 0.228.

Table 1
Result of Analysis of mean levels of TNF-α and IL-6

Variable	Group					*p*
	K1 N=6 Mean	K2 N=6 Mean	K3 N=6 Mean	K4 N=6 Mean	K5 N=6 Mean	
TNF-α levels (pg/ml)	83.36	246.47	155.83	171.80	195.77	
Std deviation	23.73	43.21	36.38	22.50	47.67	
Shapiro Wilk	0.209*	0.664*	0.629*	0.615*	0.239*	
Levene test						0.409**
One-way ANOVA						0,000***
IL-6 levels (pg/ml)	33.83	115.11	58.85	76.70	78.05	
Std deviation	8.55	16.51	19.71	6.03	17.33	
Shapiro Wilk	0.378*	0.557*	0.257*	0.884*	0.353*	>0.05*
Levene test						0.228**
One-way ANOVA						0.000***

Note: *normal p>0.05; ** homogeneous p>0.05; ***significant p<0.05

Table 2
Descriptive Analysis, Normality, Homogenity, and Comparasion of TNF-α Levels between groups

	Group					*p*
	K1	K2	K3	K4	K5	
Mean±SD	83.36± 23.73	246.47± 43.21	155.83 ± 36.38	171.80 ± 22.50	195.77 ± 47.67	
*Shaphiro wilk**	0.209	0.664	0.629	0.615	0.239	
Levene						0.409
One way anova						0.000

*p-value, SD: standard deviation

Table 2 shows that from the descriptive analysis, the K2 group shows the highest TNF-α level of 246.47±43.21 pg/mL while the K1 group shows the lowest TNF-α level of 83.36±23.73 pg/mL. Among the rats treated with Habbatussauda, TNF-α levels tended to increase with the high dose of Habbatussauda given. Based on the results of the normality analysis of the distribution of data tested with Shapiro Wilk, it was found that each group had a normal distribution of TNF-α level data, indicated by a p > value of 0.05. The results of the homogeneity analysis of the variant tested by the Levene test also showed that the homogeneous results were shown with a p value of 0.409 or p>0.05.

The requirements of the parametric differential test >2 groups were thus met so that the One Way Anova test was used to see the significance of the difference in TNF-α levels between the five groups of rats. Based on the results of the one-way anova test, a value of p<0.001 was obtained so that it was stated that there were at least two groups of rats with meaningfully different levels of TNF-α. A test of the difference between the two groups is necessary to find out which group pairs have meaningfully different TNF-α levels. The difference between these two groups was carried out by the post hoc LSD test, and the results are presented in table 3 below:

Table 3
Analysis of differences in TNF-α level (pg/mL) Between groups

Group Comparison	Average difference (pg/mL)	p
K1 vs K2	-178,10*	0,000
K1 vs K3	-72,47*	0,002
K1 vs K4	-88,44*	0,000
K1 vs K5	-112,40*	0,000
K2 vs K3	105,63*	0,000
K2 vs K4	89,67*	0,000
K2 vs K5	65,70*	0,004
K3 vs K4	-15,97	0,451
K3 vs K5	-39.93	0,067
K4 vs K5	-23,97	0,262

*= meaningful difference

Table 3 shows that almost all group pairs showed significant differences in TNF-α levels (p<0.05); except for the K3 vs K4 and K5 group pairs, with the K4 vs K5 group (p>0.05). TNF-α levels in K2, K3, K4 and K5 clusters were significantly higher than K1. Higher levels of TNF-α in K2 than in K1 suggest that induction of a high-fat diet has an impact on increasing TNF-α levels.

TNF-α levels in the K3, K4 and K5 groups which were significantly lower than in K2 can be interpreted that the administration of Habbatussauda has the effect of reducing TNF-α levels in Wistar rats induced by a high-fat diet. Meanwhile, the comparison of TNF-α levels between K3, K4 and K5 showed that increasing the dose of Habbatussauda did not have a different effect on TNF-α levels. The effectiveness of Habbatussauda between doses of 0.2 mL; 0.4 mL; and 0.8 mL/200 gBB was relatively similar in lowering TNF-α levels in Wistar rats induced by a high-fat diet.

The comparison of TNF-α levels between the two groups can also be seen from the following graph:

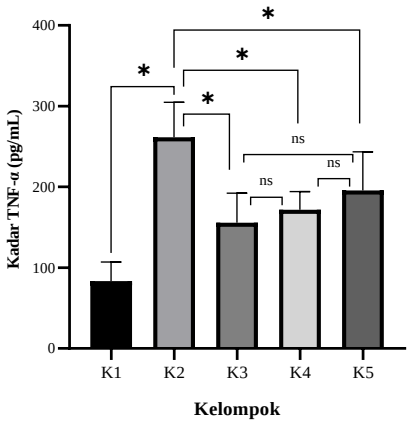

Figure 1. Description and Comparison of Tnf-A Levels Between Groups

Table 4
Descriptive Analysis, Normality, Homogeneity And Comparison Of IL-6 Levels Between Groups

	Group					*p*
	K1	K2	K3	K4	K5	
Mean±SD	33.83 ±8.55	115.11± 16.51	58.85 ±19.7 1	76.70 ±6.03	78.05 ±17.3 3	
*Shaphiro wilk**	0.378	0.557	0.257	0.884	0.353	
Levene						0.228
One way anova						<0.001

*p-value, SD: standard deviation

Table 4 shows that from the descriptive analysis, the highest IL-6 level is also in the K2 group, which is 115.11±16.51 pg/mL and the lowest in the K1 group, which is 33.83±8.55 pg/mL. Among the rat group treated with Habbatussauda, IL-6 levels also tended to increase along with the high dose of Habbatussauda given. Based on the results of the normality analysis of the distribution of data tested with Shapiro Wilk, it was found that each group had a normal distribution of IL-6 levels shown with a p > value of 0.05. The results of the variant homogeneity analysis tested by the Levene test also showed homogeneous results shown with a p value of 0.228 or p>0.05.

The significance of the difference in IL-6 levels between the five groups of rat was then tested with one-way anova and a value of p<0.001 was obtained so that it was stated that there were at least two groups of rat with significantly different IL-6 levels. The test between the two groups with the LSD post hoc test was then carried out and the results were obtained as presented in the table:

Table 5 shows that all group pairs showed significant differences in IL-6 levels (p<0.05); except for the K4 vs K5 group pairs (p>0.05). IL-6 levels in K2, K3, K4 and K5 clusters were significantly higher than K1. Higher levels of IL-6 in K2 than in K1 suggest that induction of a high-fat diet has an impact on increasing IL-6 levels.

IL-6 levels in the K3, K4 and K5 groups were significantly lower than in K2, indicating that the administration of Habbatussauda also had the effect of lowering IL-6 levels in Wistar rats induced by a high-fat

diet. However, the effect of the decrease shown is not optimal because IL-6 levels in the K3, K4 and K5 groups are still higher than in K1.

Table 5
Analysis Of Differences In Il-6 (Pg/mL) Levels Between Groups

Group Comparison	Average Difference (pg/mL)	p
K1 vs K2	-81.28*	<0.001
K1 vs K3	-25.03*	0.007
K1 vs K4	-42.77*	<0.001
K1 vs K5	-44.22*	<0.001
K2 vs K3	56.26*	<0.001
K2 vs K4	38.51*	<0.001
K2 vs K5	37.06*	<0.001
K3 vs K4	-17.75*	0.046
K3 vs K5	-19.20*	0.032
K4 vs K5	-1.45	0.865

*= meaningful difference

IL-6 levels in K4 and K5 which were significantly higher than K3 showed that the administration of Habbatussauda at a dose of 0.001 mL; and 0.002 mL/gBB provided a lower IL-6 level reduction effect than a dose of 0.001 mg/gBB in high-fat diet-induced Wistar rats. IL-6 levels in the K4 and K5 groups that were not meaningful showed that the administration of Habbatussauda at a dose of 0.002 mL; and 0.004 mL/gBB provided a relatively similar effect of reducing IL-6 levels in Wistar rat induced by a high-fat diet. From the comparison of IL-6 levels between K3, K4 and K5 groups, it was found that K3 was the best compared to K3 and K5.

The comparison of IL-6 levels between the two groups can also be seen from the following graph:

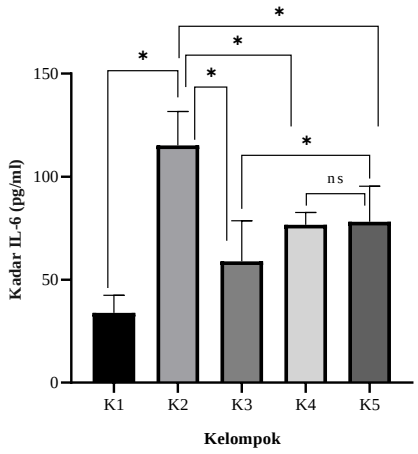

Figure 2. Description and Comparison of Il-6 Levels Between Groups

Discussion

A high-fat diet can cause a condition of hypercholesterolemia in which cellular cholesterol homeostasis regulation will occur starting from absorption, intracellular metabolism and cholesterol depletion (Chang *et al.*, 2015)'(Pei *et al.*, 2017). The imbalance that occurs causes the conversion of macrophages, mesangial cells and vascular smooth muscle cells into foam cells (Pei *et al.*, 2017). The main transporters responsible for cholesterol depletion are SR-B1, ABCG1 and ABCA1. The ABCG1 and ABCA1 genes are regulated by *the liver X receptor* (LXR). It will further activate PPARγ and promote cholesterol excretion through LXR and reduce inflammation by inhibiting NF-kB (Fangbo et al., 2023). A high-fat diet also causes an increase in the levels of free fatty acids from adipocytes into the blood which will activate CCP which will affect the expression of IKK, JNK and p38 MAPK, thereby stimulating the release of inflammatory factors such as TNF-α and IL-6 (Chang *et al.*, 2015).

A high-fat diet can also change the composition of the gut microbiota by reducing microbial diversity and reducing fiber-breaking microbes, thereby increasing the risk of obesity. An increase in the number of Enterobacteriaceae bacteria causes LPS lipid A to react strongly to TLR-4 in intestinal tissue, causing an inflammatory response through the activation of the TLR-4/MyD88/NF-kB signaling pathway. Activation of these pathways causes an inflammatory response in the form of increased vascular permeability in the intestine and increased serum levels of TNF-α and IL-6. The crude fiber contained in Habbatussauda can increase the diversity of intestinal microbiota and the production of SCFA in the colon, so that it can increase the metabolic rate and thermogenesis (Chang *et al.*, 2015).

The results showed that administration of Habbatussauda at doses of 0.001 mL/gBB, 0.002 mL/gBB and 0.004 mL/gBB could reduce TNF-α and IL-6 levels in rats fed a high-fat diet. Research by Alkinrinde *et.al* on the effect of *Nigella sativa* oil given an oral dose of 1 ml/kg for 7 consecutive days in rats fed CdCl2 can reduce TNF-α and IL-2 levels (Akinrinde *et al.*, 2022). Another study by Khazaei *et al.* by conducting research on the effect of Nigella sativa on inflammation-induced myocardial fibrosis in male rats was able to reduce TNF-α, IL-6, MDA levels and increase SOD and catalase levels with a dose of *Nigella sativa* 400 mg/kg/day intraperitonel (Khazaei *et al.*, 2017).

Nigella sativa has antihyperlipidemic effects because it contains *thymoquinone* and other active components (Abbas, 2018). Habbatussauda peroral oil at a dose of 2 mL/kg contains 5% thymoquinone. Thymoquinone can lower LDL, total cholesterol, TG and decrease HDL levels. Thymoquinone can exert effects in promoting hepatic arylesterase activity, regulation of genes that affect cholesterol metabolism (Apo-A1, Apo-B100, and LDL receptor genes), and has antioxidant properties through ROS modulation (Abdelrazek *et al.*, 2018). Thymoquinone in *Nigella sativa* can also lower lipid levels through the regulation of the HMG-CoA reductase gene and inhibit the peroxidation of non-enzymatic lipids in liposomes, and work as a carrier of various reactive oxygen species including superoxide anions and hydroxyl radicals (Abbas, 2018) *Nigella sativa* also contains fiber which will affect the decrease in cholesterol absorption and increase the synthesis and degradation of bile acids (Abbas, 2018).

Nigella sativa contains polysaccharides which are groups of carbohydrates formed from monosaccharide units that join together through glycosidic relationships (α and β configurations) and have biological activities such as anti-inflammatory and antioxidant (Trigui *et al.*, 2018). The flavonoids in Nigella sativa can decrease cholesterol synthesis, suppress ROS and maintain the antioxidant

defense system. Flavonoids also improve the efficiency of liver cells to remove LDL from the blood circulation by increasing the density of LDL receptors in the liver and binding to apoliporotein B (Abbas, 2018).

Habbatussauda contains *Thymoquinone* which has a protective effect against progressive lipid deposition induced by hypercholesterolemia, as an anti-inflammatory, antioxidant and piroptosis (Chang *et al.*, 2015)ˈ(Safi *et al.*, 2021). The antioxidant properties *of thymoquinone* are shown by a decrease in MDA, oxLDL and fibronectin levels (Shafik *et al.*, 2015). *Thymoquinone* can lower triglyceride levels by decreasing the activity of HMG-CoA reductase hepatic, increasing arilesterase activity, regulating genes that affect cholesterol metabolism and lowering oxidative stress (Shahbodi *et al.*, 2021). *Thymoquinone* can also have an agonist effect on PPAR-γ and increase the regulation of LDL receptors in hepatocytes so that cholesterol removal, and suppression of the HMG-CoA R gene and cholesterol synthase (Hadi et al., 2021).

Hypercholesterolemia can increase excessive ROS by sending signals to downstream ROS-sensitive signaling pathways, such as NF-κB, ERK1/2, p38 MAPK, and autophagy-related signaling to induce, resulting in increased levels of pro-inflammatory cytokines (TNF-α and IL-6. TQ contained in Habbatussauda can inhibit ROS so that it inhibits signaling activation and ultimately lowers the levels of pro-inflammatory cytokines (TNF-α and IL-6) (Abbas, 2018).

CONCLUSION

Habbatussauda oil at a dose of 0.001 mL/gBW, 0.002 mL/gBW, and 0.004 mL/gBW for 14 days can reduce levels of TNF-α and IL-6 in rat induced by a high-fat diet. The levels of TNF-α and IL-6 between groups K3, K4, and K5 showed that the dose of Habbatussauda K3 was the best compared to K4 and K5. Habbatussauda oil has a spicy taste, causing a burning sensation in the epigastrium which irritates the stomach, so when giving habbatussauda oil you need to consider the time of giving habbatussauda and the dose that is safe for the stomach.

SUGGESTION

It is necessary to add a positive control group with functions almost similar to Habbatussauda (*Nigella sativa*), such as fibrat drugs, so that the difference in effects between the two can be seen. It is necessary to test the composition of Habbatussauda first before using it in the next research.

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