Oral Administration of Perilla (Perilla frutescens) Leaves Extract Inhibited the Increase of Tumor Necrosis Factor-Alpha, Interleukin-6 and Did Not Inhibit the Increase of Foam Cell Amount in Obese Wistar Rats (Rattus norvegicus)

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Abstract

Background: Obesity is a disorder involving excessive fat accumulation which increases the formation of reactive oxygen species that leads to inflammation and atherosclerosis. Perilla (Perilla frutescens) leaves possess anti-inflammatory effects and is rich in antioxidants, such as terpenoid, carotenoid, phenol, and flavonoid. The purpose of this study was to prove whether the administration of perilla (Perilla frutescens) leaves extract may have an inhibitory effect towards the build-up of TNF-alpha, IL-6, and foam cell in obese Wistar rats.
Methods: This research was an experimental randomized post-test only control group design. Samples were 34 obese male rats (Rattus norvegicus) aged 6-8 weeks. The samples were divided into 2 groups, namely the control group which was obese rats administered with high fat diet and placebo (distilled water), and the treatment group which was obese rats allotted with high fat diet and perilla (Perilla frutescens) leaves extract with a dose of 500 mg/kg rat weight for 4 weeks. TNF-alpha and IL-6 were measured by ELISA method from rat blood serum, foam cell was examined by histopathology examination from the ascending aorta. The research data was analyzed using T-independent test.
Results: The results showed that there was a significantly lower outcome in the mean level of TNF-alpha, the mean of control group was 80.09±1.64 pg/mL and the treatment group was 60.40±1.35 pg/mL ($p<0.001$). Similarly, there was a significantly lower results in the mean level of IL-6, the mean of control group was 0.67±0.00 pg/mL and the treatment group was 0.51±0.08 pg/mL ($p<0.05$). There was no significant difference in the mean level of foam cell, the p value was 0.215 even though the mean level of foam cell in control group was 1.78±2.60 cells/field of view and in treatment group was 0.70±1.72 cells/field of view.
Conclusions: From the outcomes of this study, it could be concluded that oral administration of perilla (Perilla frutescens) leaves extract significantly inhibited the increase of TNF alpha levels and IL-6 levels, but did not inhibit the increase of foam cell amount in obese Wistar rat.

Keywords: foam cell; IL-6; obese; perilla leaves extract; TNF- α

1. Introduction

There are several factors that can affect the aging process, such as free radicals, changes in hormone levels,

decreased immune system, unhealthy lifestyle to stress factors. Consumption of high-fat food and reduced physical activity will lead to weight gain leading to obesity (Reiss et al., 2020). Obesity is now often found around the world and is starting to replace malnutrition as a health problem (Nugroho et al., 2018). According to the Basic Health Research Report (Riskesdas) in 2018, the prevalence of obesity increased to 21.8% where previously in 2013 it was only 14.8% (Zalukhu and Lubis, 2020). Obesity will increase the production of reactive oxygen species (ROS) which causes a decrease in sources of antioxidants in the body such as superoxide dismutase (SOD) and catalase (CAT) (Rahmawati, 2014). Increased ROS causes oxidative stress resulting in oxidative damage to cells, tissues and organs. (Susantiningsih, 2015). Increased oxidative stress causes inflammation thereby increasing the production of proinflammatory cytokines produced by adipocytes and preadipocytes, namely TNF-α and interleukin 6 (Sharif et al., 2020, Pal et al., 2014). Inflammation in obesity results in endothelial dysfunction associated with atherosclerosis. Foam cells play an important role in the formation and development of atherosclerosis. (Syaputra et al., 2014).

Several treatments for obesity have been researched and implemented, from lifestyle changes, dietary changes, physical activity, drugs, surgery to herbal remedies (Zalukhu and Lubis, 2020). One type of vegetable that is believed to have anti-inflammatory and antioxidant benefits is perilla leaves which contains many terpenoid compounds. Terpenoids are able to suppress the activation of pro-inflammatory cytokines (Parawansah et al., 2022). Another content that is mostly found in perilla leaves is phenolic compounds, which show potential as antioxidants. One part of the phenol which is also found in perilla leaves is flavonoids that can reduce lipid accumulation in adipose tissue and inhibit lipid peroxidation by reducing malondialdehyde (MDA) serum levels in rats fed a high-fat diet (Hou et al., 2022). Flavonoid compounds inhibit the activation of NF-kB and related pathways that can prevent an increase in pro-inflammatory cytokines, including TNF-α and IL-6 (Szostek et al., 2021).

The antioxidant effect of perilla leaves works by increasing antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) with scavenging reactive oxygen species (ROS). Increased antioxidant enzymes inhibit chronic inflammation by inhibiting the production of inflammatory cytokines, namely TNF α and interleukin 6 (Lee and Han, 2012). Based on the description above, it can be seen that the active substances in perilla leaves have many health benefits, especially for preventing degenerative diseases due to obesity which in turn can prevent the aging process and increase life expectancy. The researcher wants to examine the effect of the administration of perilla leaves extract in inhibiting the increase in levels of TNF-α, interleukin-6, and the number of foam cells in obese rats.

2. Methods

2.1. Study Design and Experimental Animals

This research is a true experimental method with a randomized posttest only control group design. Production of perilla leaves extract was carried out at the Postharvest Engineering Laboratory, Faculty of Agricultural Technology, Udayana University. The experiment was carried out at the Integrated Biomedical Laboratory Unit of the Faculty of Medicine, Udayana University. Histopathological examination was carried out at Sentra Laboratory, Denpasar. Examination of TNF-α and IL-6 levels was carried out at the Integrated Biomedical Laboratory Unit, Division of Biochemistry and Molecular Biology, Faculty of Medicine, Udayana University. The sample needed in this experiment was 34 male Wistar rats (n=16), male, 6-8 weeks with Lee index >0,30. 34 rats divided into 2 groups: control and treatment group. This research has been approved by the ethics commission of Udayana University, Bali, (B/79/UN14.2.9/PT.01.04/2022).

2.2. Perilla leaves Extract Production

Perilla leaves were washed and dried at temperature room, then weighed and crushed into a fine powder and filtered using mesh 70. The powder was soaked in 60 ml of 95% ethanol for 1 hour and filtered using Whatman no 4. The residue was extracted again with 30 ml of 95% ethanol for 1 hour. The evaporation was done using a rotary evaporator at 40°C. AB-8 macroporous was added to remove sugar on extracts. The extract was stored in airtight container at 4°C (Zhao et al., 2019).

2.3. Experimental Animal Treatment

34 rats were adapted for 7 days before being fed with high fat diet by administering high cholesterol diet suspension (mixing lard and duck egg yolk). The high fat diet was given for 4 weeks by mixing standard feed 47 brand BR-II and high cholesterol diet suspension. Drinking water was mixed with 8% corn syrup ad libitum. After 28 days, the rats were examined and were said to be obesity if Lee index >0.3. The rats were divided randomly into two groups namely the control group, administered with distilled water and high-fat diet, and the treatment group which was given perilla leaves extract and high-fat diet.

2.4. TNF-α and IL-6 Level Examination

Examination of TNF-α and IL-6 level was done using the BT LAB brand ELISA kit, with steps as follow: Insert the strip into the mold for use. 50µl standard was added into the standard well and 40µl sample into the sample well. Then 10µl of mouse TNF-alpha antibody, for TNF-α examination, and 10µl of mouse IL-6 antibody, for IL-6 examination, each was added to the sample well and 50µl of streptavidin-HRP was added to the sample wells and standard wells. The ingredients were mixed well and covered with sealer, then incubated for 60 minutes at 37˚C. The plate was washed 5 times using wash buffer. The wells were moistened with 300µl of wash buffer for 30 seconds to 1 minute each wash. 50µl of each substrate A and B solution was added to each well. The plate was incubated for 10 minutes at 37˚C in the dark. 50µl of stopping solution was added to each well, changing the color to blue yellow. The optical density (OD value) of each well was determined as soon as possible using a microplate reader set at 450 nm after 10 minutes of stopping solution.

2.5. Foam Cells Count Examination

Examination of the number of foam cell was done by taking a sample of the aorta from mice that were previously euthanized. The histopathological examination was done using Hematoxylin Eosin staining (HE) (Syaputra et al., 2014). Foam cells were counted using digital analysis method. In the analysis, an Olympus CX21 light microscope was used. Each sample was photographed with 40x and 400x magnification using the Optilab Viewer 2.2 software. The number of foam cells was calculated at 400x magnification in 9 fields of view and then the average for each preparation was calculated to obtain the number of foam cells in units of cells/ field of view (FOV). Calculation of the number of foam cells was done using Image software Raster 3.0.

2.6. Statistical Analysis

Statistical analysis was performed with SPSS. Normality test was assessed using Shapiro-Wilk test and homogeneity test was assessed with Levene's test. Comparability test was assessed using t-independent for normally distributed data. While data transformation was performed for non-normally distributed data and then Mann-Whitney nonparametric test was used.

3. Results

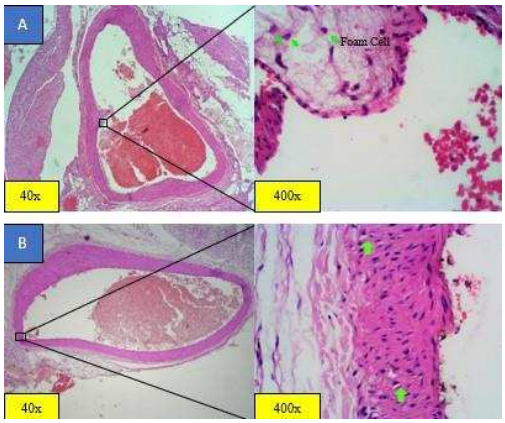

Fig 5. Histology of Foam Cell in Aorta with Hematoxylin Eosin Staining at 40x and 400x magnification

Description:

Figure A: A cross-sectional view of the ascending aorta in the control group which was given a high-fat diet and distilled water. At 40x magnification the entire aortic wall is visible. It appears the formation of plaque, which is thickening of the tunica intima with accumulation of fat. At 400x magnification, a picture of several foam cells appears, namely foamy cytoplasm with a round nucleus of purplish-blue color.

Figure B: A cross section of the ascending aorta in the treatment group which was given a high-fat diet and extracts. At 40x magnification, blood vessel walls were thickened, no plaque was visible and at 400x magnification, the smooth muscle cell proliferation and some foam cell in tunica intima was visible, i.e eosinophilic cytoplasm with round purplish blue colored nuclei and elastin fibers were visible in tunica adventitia.

Normality test was done using Shapiro-wilk test and is presented on Table 1. Based on Table 1, it was concluded that the data of TNF-α level was normally distributed ($p > 0.05$). The data for IL-6 and foam cell levels had been transformed into log10 data and the results were non-normally distributed ($p<0.05$), so the non-parametric Mann Whitney test was carried out.

Table 1. Normality Test

Variables	Group	n	Post-test	Desc
TNF-α (pg/mL)	K	17	0.287	Normal
	P	17	0.104	Normal
IL-6	K	17	0.005	Non Normal
(pg/mL)	P	17	0.003	Non Normal
Foam Cell	K	14	0.000	Non Normal
(cells/FOV)	P	17	0.000	Non Normal

K: control group; P: treatment group; The number of control groups in the foam cell variable is 14 samples, after deducting the extreme values in 3 samples to prevent bias in the data

Levene's homogeneity test is presented in Table 2. Based on Table 2, it was concluded that the data was homogeny ($p < 0.05$). Homogeneity test was not carried out on IL-6 and foam cell because the data was non-normally distributed.

Table 2. Homogeneity Test

Variable	F	p	Desc
TNF-α	0.871	0.358	Homogeny

Comparability test on TNF-α level between groups was done using t-independent test and is presented in Table 3.

Table 3. Comparability Test of TNF-α

Variable	Group	n	Mean	SD
TNF-α	K	17	80.09	1.64
	P	17	60.40	1.35

K: control group; P: treatment group

In the results of the analysis using the t-independent test there was a significant difference in the mean TNF-α levels between groups ($p \leq 0.05$). The treatment effect test obtained a value of 0.9, so there was a large difference in TNF-α levels between the control and treatment groups. The results of the analysis can be seen in table 4.

Table 4. Comparison of TNF-α between Groups

Variable	Groups	Mean	t	p	Desc
TNF-α	K-P	19.68	38.12	<0.001	Significantly differrent

In the results of the analysis of the normality test for IL-6 and foam cell levels in table 5, the data results were non-normally distributed ($p < 0.05$). Data transformation was carried out but the data was still non-normally distributed, so Mann-Whitney test was carried out and the result is presented in Table 6.

Table 6. Non-Parametric Test for IL-6 and Foam Cells

Variables	p
IL-6	0.000
Foam Cells	0.215

In the analysis using the Mann Whitney test, there was a significant difference in the mean IL-6 level ($p<0.05$) between groups and no significant difference was found in the number of foam cells between groups ($p>0.05$).

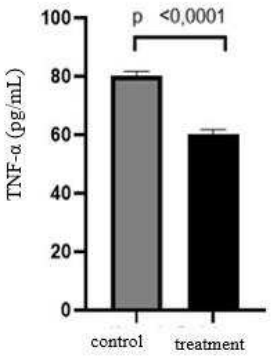

Fig 1. Comparison of Mean TNF-α Level between Groups

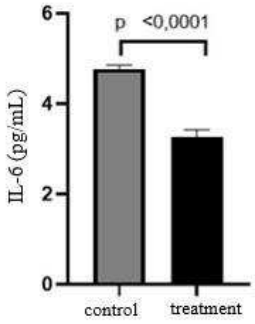

Fig 2. Comparison of Mean Differences in IL-6 between Groups

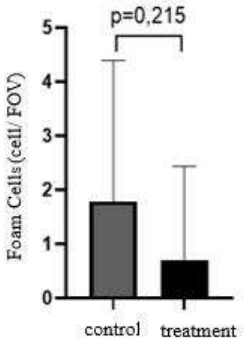

Fig 3. Comparison of Mean Differences in Foam Cells between Groups

4. Discussion

4.1. Effect of Perilla leaves Extract (Perilla frutescens) on TNF-alpha

Carotenoids in perilla leaves extract can reduce inflammation induced by oxidative stress generated in adipose tissue, associated with the pathogenesis of obesity. In the previous study conducted by Waldeck et al., carotenoid and terpenoid compounds have been found in perilla leaves (Waldeck et al., 2010). Carotenoids attenuated oxidative stress-induced inflammation through a decrease in the adipokines monocyte chemoattractant protein-1 (MCP-1) which in turn inhibited the activation of NF-kB, activator protein-1 (AP-1), and signal tranducer and activator of transcription 3 (STAT3). These effects lead to a decrease of TNF-alpha production (Roman et al., 2021). In addition, a study by Asif reported that the phytochemical test contained phenol and flavonoids in Perilla frutescens (Asif, 2012). Flavonoids in perilla leaves extract inhibit other TNF-alpha signaling pathway, i.e mitogen -activated protein kinase (MAPK) and c-JUN N terminal kinase (JNK) which then can reduce the expression of TNF-alpha (Tantipaiboonwong et al., 2021). This is in line with the phytochemicals test results of perilla leaves extract conducted at UPT Analytical Laboratory, Udayana University Bali, with carotenoids 11,518.6733 mg/100g, terpenoids were detected, flavonoids 9,961.77 mg/100g, and phenol 3,405.02 mg/100g. When compared to a study by Straumite et al., using the peppermint leaf extract (Mentha piperita L.), the carotenoid content was 10.3 mg/100g, which was lower than perilla leaves (Apriliyani et al., 2021) In this study, there was a significant decrease in TNF-alpha levels between the control group and the treatment group.

4.2. Effect of Perilla leaves Extract (Perilla frutescens) on IL-6

The terpenoid compounds found in perilla leaves are able to suppress the activation of pro-inflammatory cytokines by inhibiting the expression of COX-2 and iNOS enzymes which are responsible for increasing proinflammatory cytokines, including IL-6 (Merhan, 2017). Flavonoids in perilla leaves are able to inhibit the secretion of IL-1β, which is the main cytokine that can induce IL-6 which in turn can cause an inflammatory response (Zhang et al., 2014). In addition, it was reported that carotenoids which are also found in perilla leaves can reduce the proinflammatory cytokine IL-6 caused by a high-fat diet (Olatunde et al., 2020). Studies showed that carotenoids are able to inhibit IL-6 release by inhibiting Nf-kB activation and suppressing the AP-1 transcription factor (Roman et al., 2021). In the other hand, carotenoids serve as antioxidants, they inhibit oxidative stress by transferring electron and hydrogen atom (Olatunde et al., 2020). This is in line with the IC50 test results of perilla leaves extract, with IC50 value of 58.90 ppm that shows a strong antioxidant activity.

4.3. Effect of Perilla leaves Extract (Perilla frutescens) on the Number of Foam Cells

Carotenoids in perilla leaves are able to inhibit atherogenesis by inhibiting the formation of foam cells thereby reducing the occurrence of heart disease. Carotenoids can significantly inhibit cholesterol esterification during the formation of foam cells in macrophages and inhibit macrophage phagocytosis (Relevy et al., 2014). Phenol and flavonoid compounds have antioxidant effect that works by preventing LDL oxidation and triggering the efflux of cholesterol from macrophages which play a role in the formation of foam cells (Waldeck et al., 2010). In the research by Syaputra et al., a significant number of foam cells were found in all samples administered with high fat diet for 12 weeks (Syaputra et al., 2014). In this study, it was found that the results of the number of foam cells were not consistent between samples. This may be due to the shorter duration of administration of the high-fat diet.

4.4. Perilla leaves Extract (Perilla frutescens) and Anti-Aging Medicine

As previously explained, based on the results of a phytochemical screening, perilla leaves extract contains terpenoids, carotenoids, phenols and flavonoids. The content of this active substance is an antioxidant that can fight free radicals. Free radicals that increase in obesity cause oxidative stress and increase the production of pro-inflammatory cytokines, TNF-α and IL-6. On the other hand, it also causes the process of atherosclerosis which begins with the formation of foam cells. By knowing the dangers of increased free radicals in obesity, it is hoped that we can avoid them, so that aging can be prevented and inhibited.

5. Conclusion

Administration of oral perilla leaves (Perilla frutescens) extract inhibits the increase in TNF-α and IL-6 level, but not the number of foam cells in obese Wistar rats (Rattus norvegicus).

Acknowledgement

This paper presents Oral Administration of Perilla (Perilla frutescens) Leaves Extract Inhibited the Increase of Tumor Necrosis Factor-Alpha, Interleukin-6, and Did Not Inhibit the Increase of Foam Cell Amount in Obese Wistar Rats (Rattus norvegicus).

Deepest gratitude to God Almighty for enabling me to finish this paper. Appreciation to dr.Agus Eka Darwinata,S.Ked, PhD and Prof.dr.I Gusti Made Aman, Sp.FK for being my first and second advisor throughout every process of writing this paper.

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