Reduction of Oxidative Stress in Dyslipidemic Sprague Dawley Rats with Exposure to Mulberry Leaf Extract and Simvastatin

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ABSTRACT

Dislipidemia menyebabkan stress oksidatif yang menyebabkan akumulasi subendotel dari lipoprotein aterogenik serta memicu pembentukan radikal bebas. Senyawa metabolit sekunder seperti flavonoid pada daun murbei dapat bertindak sebagai antioksidan yang mampu menekan produksi radikal bebas penyebab stres oksidatif. Tujuan dari penelitian ini adalah untuk menilai pengaruh ekstrak daun murbei dan statin terhadap kadar MDA pada tikus Sprague Dawley dislipidemia. Penelitian ini berjenis true experimental dengan pre-post test with control group design. Sampel dalam penelitian ini adalah daun murbei dan tikus berjenis Sprague dawley. Daun murbei dilakukan ekstrasi menggunakan metode maserasi dengan pelarut etanol 70% kemudian dilanjutkan pengujian kadar flavonoid total dan pengujian antioksidan menggunakan DPPH. Jumlah sampel pada penelitian ini yaitu 30 tikus jantan yang dibagi menjadi 5 kelompok yaitu kelompok P1 (HFD + ekstrak daun murbei), P2 (HFD + simvastatin), P3 (HFD + ekstrak daun murbei + simvastatin) serta Kelompok normal dan kelompok kontrok negatif. Ekstrak etanol daun murbei memiliki nilai IC50 sebesar 5.61 ppm yang termasuk kategori sangat kuat. Intervensi dilaksanakan selama 8 minggu. Kadar MDA diperiksa sebelum dan setelah intervensi. Hasil menunjukkan adanya penurunan signifikan kadar MDA pada kelompok perlakuan P1, P2 dan P3. Perubahan kadar MDA antar kelompok diuji dengan one way ANOVA dengan hasil yang signifikan ρ=0,000. Pemberian ekstrak daun murbei yang kaya flavonoid dan simvastatin secara signifikan menurunkan kadar MDA dalam darah tikus.

Keyword: MDA; Daun Murbei; Simvastatin; Flavonoid

ABSTRAK

Dyslipidemia induces oxidative stress, leading to the subendothelial accumulation of atherogenic lipoproteins and the initiation of reactive oxygen species (ROS) formation. Secondary metabolites such as flavonoids found in mulberry leaves can act as antioxidants, suppressing the production of free radicals responsible for oxidative stress. This study aims to evaluate the impact of mulberry leaf extract and simvastatins on MDA levels in dyslipidemic Sprague Dawley rats. This research is a true experimental type with a pre-post test with control group design. The samples in this study were mulberry leaves and Sprague Dawley rats. Mulberry leaves were extracted using the maceration method with 70% ethanol solvent, then continued with total flavonoid content testing and antioxidant testing using DPPH. The number of samples in this study was 30 male rats which were divided into 5 groups; group P1 (HFD + mulberry leaf extract), P2 (HFD + simvastatin), P3 (HFD + mulberry leaf extract + simvastatin) as well as the normal group and the negative control group. Mulberry leaf ethanol extract has an IC50 value of 5.61 ppm which is very strong category. The intervention was carried out for 8 weeks. MDA levels were checked before and after intervention. The results showed a significant decrease in MDA levels in treatment groups P1, P2 and P3. Changes in MDA levels between groups were tested using one way ANOVA with significant results p=0.000 (p < 0,05). Administration of mulberry leaf extract which is rich in flavonoids and simvastatin significantly reduces MDA levels in the blood of rats.

***Kata Kunci: :** MDA; Mulberry Leaves; Statins; Flavonoids*

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Introduction:

Dyslipidemia is a disorder of lipid metabolism resulting from the interplay of genetic and environmental factors, characterized by elevated levels of total cholesterol, triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and reduced levels of high-density lipoprotein cholesterol (HDL-C) (Hidayatullah et al., 2022). Dyslipidemia can lead to significant clinical conditions, including cardiovascular diseases (CVD). Heart disease cases in Indonesia reached 12.93 million in 2021 and increased to 15.5 million in 2022 (Lestari, 2023).

Elevated lipid profiles, including increased triglyceride, LDL-C levels and decreased levels of HDL-C, can lead to oxidative stress (Wengrofsky et al., 2019). Oxidative stress is characterized by the buildup of free radicals, which subsequently enhances lipid peroxidation, producing the metabolite known as *malondialdehyde* (MDA) in the bloodstream. MDA can serve as an indicator of the extent of oxidative stress in the body (Muhajirin & Marjan, 2019).

Mulberry (*Morus alba* L.) is a wild plant that can be cultivated in tropical and subtropical climates. Mulberry leaves contain chemical compounds flavonoids, alkaloids, terpenoids, saponins and tannins (Purnama, 2022). Mulberry leaves provide a natural source of nutrients and antioxidants that can be advantageous for humans. Earlier research has indicated that mulberry leaf extract contains higher levels of total phenols and flavonoids compared to the fruit (Hilwiyah et al., 2015).

Consuming foods containing flavonoids can serve as a strategy to mitigate the risk of oxidative stress and its adverse effects on the human body. When rats are fed a high-fat diet (HFD), there can be increases in lipid peroxidation products like MDA levels and other protein oxidation products (Lasker et al., 2019). Earlier research has indicated that hyperlipidemic rats administered mulberry leaf extract exhibited improvements in their lipid profiles and a reduction in oxidative stress (Huang et al., 2018; Peng et al., 2018). In another study, diabetic rats treated with aqueous mulberry leaf extract were able to reduce MDA levels significantly during 6 weeks of treatment (Luo et al., 2023). Dyslipidemic rats were administered simvastatin to alter their lipid profiles, and mulberry leaf extract was added to lessen the incidence of oxidative stress brought on by dyslipidemia. The synergistic effects of combining conventional medications and medicinal plants in improving lipid profile are determined by the type and quantity of medicinal plants employed in the formulation. To develop an adequate therapy protocol, thorough pharmacological study and evaluation are required.

This study aims to evaluate the effects of mulberry leaf extract and simvastatin on MDA levels in dyslipidemic *Sprague Dawley* rats.

Methods:

This research is an experimental study with pre and posttest control group design. Mulberry leaves from the Lawang Malang plantation were utilized as samples in this study, and rats were used as the experimental animals. The samples of Mulberry leaves were tested for determination at the biology lab of Ahmad Dahlan University. The mulberry leaves were subjected to extraction using the maceration method with a 70% ethanol solvent. The 1,1-*diphenyl*-2-*picrylhydrazyl* (DPPH) method is the antioxidant test that is employed to evaluate the possible antioxidant activity of the components present in the mulberry leaf methanol extract. In the DPPH test method, quercetin serves as a positive control and point of comparison. Quercetin stands out as one of the predominant flavonoid compounds found in mulberry leaves (Katsube et al., 2006).

A total of 30 male *Sprague Dawley* rats, weighing between 150-200 grams (following Federer's formula), were acquired from the PAU Nutrition and Food Laboratory, University of Gadjah Mada, Yogyakarta.

Each rats was adapted for 7 days before treatment (Rahma et al., 2017). During this adaptation phase, the rats were exclusively provided with pellet standard feed (comfeed PARs) and had ad libitum access to water. To induce dyslipidemia in the rats, following the adaptation period, all treatment groups were given *Comfeed PARs* 60% as standard feed added

with 27.8% wheat flour, 2% cholesterol, 0.2% cholic acid, and 10% lard as high-fat diet (HFD) which was given for 28 days. After confirming the development of dyslipidemia by assessing lipid profile parameters, interventions were administered to each group over an 8-week period. Subsequently, the rats were divided into five groups: treatment 1/P1 (HFD + standard feed + 600mg/kg body weight/day mulberry leaf extract), treatment 2/P2 (HFD + standard feed + 0.18mg/200g body weight/day simvastatin), treatment 3/P3 (HFD + standard feed + 300 mg/kg body weight/day mulberry leaf extract + 0.9mg/200g body weight/day simvastatin), normal group/K1 (standard feed) and negative control/K2 (HFD + standard feed). The dose of 600mg/kg is one of the optimum doses in previous research (Huang et al., 2018).

Data collection involved obtaining blood samples from the rats via the retro-orbital sinus (**figure 1**), using a 1 mL hematocrit pipette across all groups. The measurement of Malondialdehyde (MDA) levels was conducted both initially (pre-test) and final observation (post-test) after the administration of treatment.

This research received approval from the Health Research Ethics Commission (KEPK) of the Faculty of Medicine, Diponegoro University, with the reference number 23/EC-H/KEPK/FK-UNDIP/II/2023.

The results of antioxidant activity tests and total flavonoids were analyzed descriptively. Differences in MDA levels before and after intervention in each group of test animals were analyzed using the paired sample t-test. Meanwhile, for comparing MDA levels between groups, one-way ANOVA was utilized.

Fig 1. Collecting blood samples from retro-orbital

Results:
Plant Determination

A determination test was conducted on the mulberry leaf samples obtained during the first stage of the research. The findings demonstrated that it was accurate to utilize mulberry leaf samples (*Morus alba* L.) based on the identification of the sample plants' features. Determination testing on mulberry leaves was carried out at the Biology Lab at Ahmad Dahlan University.

Antioxidant Activity

The purpose of conducting the DPPH method for testing antioxidant activity is to assess the inhibitory capacity of a plant extract against DPPH free radicals. In antioxidant activity tests, quercetin serves as the standard for comparison.

Tabel 1. Antioxidant activity of Quercetin (positive control) against DPPH

No	Concentration of Quercetin (ppm)	Absorbance	% Inhibition
1	2	0,239	39,95
2	4	0,212	46,73
3	6	0,185	53,52
4	8	0,152	61,81
5	10	0,114	71,36

Tabel 2. Antioxidant activity of Mulberry Leaf Extract against DPPH

No	Concentration Mullberry Leaf Extract (ppm)	Absorbance	% Inhibition
1	2	0,246	38,19
2	4	0,218	45,23
3	6	0,204	48,74
4	8	0,174	56,28
5	10	0,168	57,79

Tables 1 and 2 demonstrate that the absorbance value obtained decreases with increasing concentrations of quercetin and mulberry leaf ethanol extract. Because quercetin and the ethanol extract of mulberry leaves captured free radicals in DPPH, the absorbance value decreased and was inversely correlated with the percentage of antioxidant activity.

According to Maulidha et al. (2015), a chemical is classified as a very strong antioxidant if its IC_{50} value is less than 50 µg/mL, strong if it is between 50 and 100 µg/mL, moderate if it is between 101 and 150 µg/mL, and weak if it is between 151-200 µg/mL.
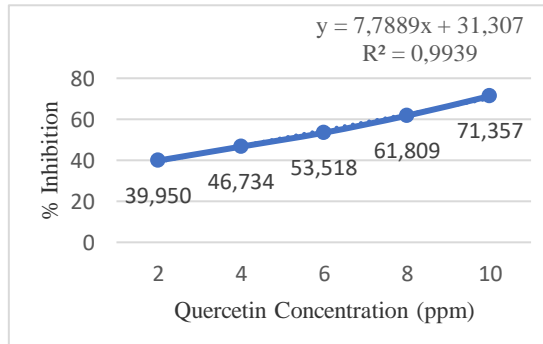

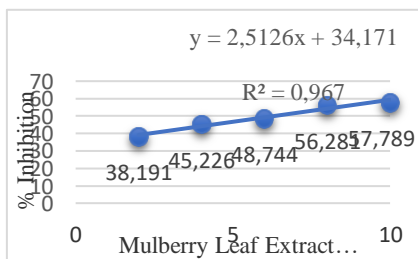Fig 2. Quercetin Free Radical Inhibition Percent Graph

Fig 3. Mulberry leaf Extract Free Radical Inhibition Percent Graph

Based on the graphs in Figures 2 and 3, we obtained a linear regression equation $y = 7,7889x + 31,307$ for Quercetin (positive control), and a linear regression equation $y = 2.5126x + 34.171$ for ethanol extract of mulberry leaves. Calculating the IC_{50} (Inhibition Concentration) value is possible using each linear regression equation that was found. The extract's 50% reduction in free radical activity (DPPH) is known as the IC_{50} value (Molyneux P, 2003). The IC_{50} values obtained can be seen in Table 3.

Table 3. IC_{50} values for Quercetin (positive control) and Mulberry Leaf Extract (*Morus alba* L)

No	Sample	IC_{50} (ppm)
1	Quercetin	4.02
2	Mulberry leaf extract	5.61

Based on the IC_{50} results, it shows that the antioxidant activity of mulberry leaf ethanol extract is as good as Quercetin as a comparison (positive control). Therefore, mulberry leaf ethanol extract has a very strong of antioxidant activity.

MDA Level

The data from the examination of MDA levels underwent a normality test using the Kolmogorov-Smirnov test. The test results indicated that the MDA level data before the intervention (pre-test) and after the intervention (post-test) in all groups followed a normal distribution ($p > 0.05$).

At the beginning of the examination. MDA levels in the normal group were measured at 1.21 ± 0.09, and these levels increased to 1.38 ± 0.09 after 8 weeks without being induced by a high-fat diet. Table 2 presents the mean MDA levels before the intervention, following induction by a high-fat diet. The data obtained for groups P1 (9.45 ± 0.08), P2 (9.60 ± 0.12), P3 (9.24 ± 0.35), and K2 (9.12 ± 0.13). After an 8-week intervention, there was a decrease in the mean MDA levels in P1 to 3.87 ± 0.12, P2 to 2.98 ± 0.10, and P3 to 2.22 ± 0.06. Meanwhile, in K2, there was an increase in the average MDA level after the intervention, reaching 9.45 ± 0.11.

Tabel 4. Results of the analysis of mean MDA levels both before and after treatment, as well as the changes observed in each group (n=30)

Group	n	Mean Pre-test ± SD	Mean Post-test ± SD	ρ-value*	Δ ± SD
Treatment 1(P1)	6	9.45 ± 0.08	3.87 ± 0.12	0.000	5.58 ± 0.31

Group	n	Mean Pre-test ± SD	Mean Post-test ± SD	ρ-value*	Δ ± SD
Treatment 2 (P2)	6	9.60 ± 0.12	2.98 ± 0.10	0.000	6.61 ± 0.35
Treatment 3 (P3)	6	9.24 ± 0.35	2.22 ± 0.06	0.000	7.02 ± 0.44
Normal (K1)	6	1.21 ± 0.09	1.38 ± 0.09	0.002	-0.17 ± 0.07
Negative control (K2)	6	9.12 ± 0.13	9.45 ± 0.11	0.016	-0.32 ± 0.20
ρ-value**					0.000

Information:
P1: Dyslipidemia (induced high fat diet), standard feed (comfeed PARS), mulberry leaf extract 600 mg/kg body weight/day
P2: Dyslipidemia (induced high fat diet), standard feed (comfeed PARS), Simvastatin 0.18 mg/200 g body weight/day
P3: Dyslipidemia (induced by high fat diet), standard feed (comfeed PARS), mulberry 300 mg/kg body weight/day and Simvastatin 0.9 mg/200 g body weight/day
K1: Normal (not induced by high fat diet), standard feed (comfeed PARS)
K2: Dyslipidemia (induced high fat diet), standard feed (comfeed PARS)
p-value*: Paired Samples T Test
p-value**: One Way ANOVA

After 8 weeks of intervention, it was observed that the highest mean MDA levels in group P3, which received the mulberry leaf extract and simvastatin intervention, significantly decreased to 7.02 ± 0.44 (p = 0.000). Additionally, in group P2, a significant reduction in mean MDA levels was also noted, measuring 6.61 ± 0.35 (p = 0.000), and in group P1, it was 5.58 ± 0.31 (p = 0.000). In the normal control group and negative control group, there was a slight increase in mean MDA levels, measuring K1 -0.17 ± 0.07 and K2 -0.32 ± 0.20, respectively.

The results of the analysis of changes in MDA levels between groups using the one-way ANOVA test showed that there was a significant difference in MDA levels between groups after the intervention was implemented (p = 0.000).

Discussion :

Dyslipidemia can indeed lead to increased lipid peroxidation. This is evident from the results of this study, where the treatment group exhibited elevated MDA levels in the blood serum of rats when compared to the normal group of rats (K1). This aligns with the findings of previous research conducted by Anitasari et al. (2017), which suggests that the increase in MDA can be attributed to an increase in total cholesterol, LDL, and triglycerides. Elevated MDA levels are indicative of heightened lipid peroxidation, indirectly signaling a higher presence of free radicals and suggesting the occurrence of an oxidative process within the cell membrane (Veskoukis et al., 2012). Increased oxidative damage may be a long-term consequence of hypercholesterolemia. Lipids serve as molecular targets for free radicals, and as a result, dyslipidemia can lead to an increase in free radicals, as represented by elevated MDA levels (Anitasari et al., 2017).

Based on the results regarding MDA levels when administering 70% ethanol extract of mulberry leaves (P1), Simvastatin (P2), and the combination (P3), all three showed significant differences in their ability to reduce serum MDA levels in the blood of dyslipidemic rats. In the case of P1, there was a notable decrease in MDA levels after treatment with a p-value < 0.05, resulting in a change of 5.58 ± 0.31. This observation aligns with the theory that suggests a strong antioxidant status is often accompanied by a reduction in MDA levels (Kresnapati et al., 2021). Research has demonstrated that mulberry leaves possess exceptional antioxidant activity. Using the DPPH method, the antioxidant activity of mulberry leaf extract was found to be 88,3 µg/mL. This is consistent with phytochemical data that show the presence of antioxidant-acting substances such flavonoids, alkaloids, saponins, tanins, and terpenoids in mulberry leaf extract (Purnama, 2022). One of the most prominent

compounds in mulberry leaves is quercetin, which is the most dominant flavonoid compound (Katsube et al., 2006). Flavonoids, functioning as antioxidants, operate by binding to free radicals, leading to the oxidation of flavonoids themselves. This occurs due to the presence of hydroxyl groups in flavonoids, characterized by high reactivity, resulting in the formation of more stable and less reactive radicals. In simpler terms, flavonoids stabilize reactive oxygen species (ROS) (Panche et al., 2016).

In group P2 there was also a significant decrease in MDA levels after treatment (p value < 0.05) with a change of 6.61 ± 0.35. Simvastatin has also been proven as an antioxidant in hyperlipidemia where the statin acts as an antioxidant against lipid peroxidation Simvastatin is a standard medication for dyslipidemia disorders. It effectively inhibits the activity of the enzyme *3-hydroxy-3-methylglutaryl-CoA reductase* (HMG-CoA reductase) and provides significant outcomes in the treatment of hyperlipidemia. Simvastatin works by inhibiting cholesterol synthesis (Aman et al., 2021). Meanwhile, in clinical and laboratory settings, other researchers have also demonstrated simvastatin's antioxidant activity, both in ischemic animal models and in patients with diabetes mellitus and dyslipidemia (Kocak et al., 2015). In the combination group (P3), there was a significant reduction in MDA levels when comparing the means before and after, with a p-value < 0.05 and a change in MDA levels of 7.02 ± 0.44. The simultaneous use of statin and mulberry leaf extract at lower concentrations can enhance the effectiveness of therapy. Several studies have indicated that simvastatin also possesses additional antioxidant effects, which may aid in protecting cells from oxidative damage. Nevertheless, simvastatin is generally not employed as the primary antioxidant but rather as a medication to address high cholesterol levels and the risk of cardiovascular disease (Mansouri et al., 2022). The most frequent side effects of statin use, elevated levels of liver and muscle enzymes, can occasionally be found by blood testing or when patients report symptoms (Cheon & Jo, 2022). Due to the antioxidant and cytoprotective properties of mulberry leaves,

previous studies have shown that adding mulberry leaf extract could protect liver tissue in rats that had been subjected to hepatotoxicity (Tag, 2015). The use of a combination of mulberry leaf extract and simvastatin is anticipated to produce a synergistic effect and reduce the occurrence of potential side effects associated with the components of simvastatin. In line with Rosenblat et al., 2013 findings simvastatin at a dose of 15 µg/ml was shown to be able to reduce oxidative stress creation by 11%, while simvastatin combined with additional pomegranate was found to be able to reduce oxidative stress formation by up to 63%. However, these models are still not being used in the actual world to examine mixes of natural products, and more research will demonstrate how applicable they are to this strategy.

MDA levels in all treatment groups showed significant differences, so that antioxidants in mulberry leaf extract and statins proved effective in reducing MDA levels in dyslipidemic rats.

Conclusions:

The administration of mulberry leaf extract, simvastatin, and their combination had an impact on reducing MDA levels in rats. The group receiving a combination of mulberry leaf extract and simvastatin demonstrated a more substantial reduction in MDA levels, measuring 7.02 ± 0.44, compared to the administration of single mulberry leaf extract or simvastatin. Lower MDA levels in the treatment groups would suggest a potential protective effect of these interventions on lipid peroxidation and oxidative stress. Further research may be required to elucidate the underlying mechanisms and clinical implications.

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