

Hyperlipidemia Preventing Activities of Standardized Ethanolic Extract of Red Spinach (*Amaranthus tricolor* L.): An in Vivo Study in Male Sprague-Dawley Rats

Aktivitas Pencegahan Hiperlipidemia dari Ekstrak Etanolik Bayam Merah (*Amaranthus tricolor* L.) Terstandar: Studi In Vivo pada Tikus Sprague-Dawley Jantan

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Abstract

Lipid metabolism disorders can lead to hyperlipidemia that triggers atherosclerosis. This study aimed to identify the potential of standardized ethanolic extract of red spinach (*Amaranthus tricolor* L.) to prevent hyperlipidemia by referring to the reduction of triglyceride level and total cholesterol in male Sprague-Dawley rats. A total of 30 experimental animals was put into 6 groups, including normal, positive control (0.9 mg/kgBW of simvastatin), negative control, treatment I (200 mg/kgBW of extract), treatment II (400 mg/kgBW of extract), and treatment III (800 mg/kgBW of extract). Preventive therapy and positive control were administered from day 1 to day 67. Hyperlipidemia was induced by feeding pure lard and duck yolk to the rats twice daily from day 8 to day 67. Determination of triglyceride level and total cholesterol was conducted on day 0 and day 67. The findings revealed that the treatment groups with ethanolic extract of red spinach at doses of 200 mg/kgBW, 400 mg/kgBW, and 800 mg/kgBW had statistically significant differences ($p < 0.05$) compared to the negative control group. The treatment III group with a dose of 800 mg/kgBW showed the most reduction of total cholesterol and triglyceride levels, which was the same as the positive control group. In conclusion, standardized ethanolic extract of red spinach possessed preventive activities against atherosclerosis based on the total cholesterol and triglyceride parameters. The highest activity was demonstrated by the dose of 800 mg/kgBW group.

Keywords: Rats; Red spinach; Hyperlipidemia; Total cholesterol; Preventive therapy; Triglyceride

Abstrak

Gangguan metabolisme lipid dapat menyebabkan hiperlipidemia yang memicu aterosklerosis. Penelitian ini bertujuan untuk mengetahui potensi ekstrak etanol dari bayam merah (*Amaranthus tricolor* L.) untuk mencegah hiperlipidemia dengan parameter penurunan kadar trigliserida dan kolesterol total pada tikus Sprague-Dawley jantan. Sebanyak 30 hewan percobaan dimasukkan ke dalam 6 kelompok, termasuk kontrol positif normal (0,9 mg/kgBB simvastatin), kontrol negatif, perlakuan I (200 mg/kgBB), perlakuan II (ekstrak 400 mg/kgBB), dan perlakuan III (800 mg/kgBB ekstrak). Terapi pencegahan dan kontrol positif diberikan dari hari ke-1 sampai hari ke-67. Hiperlipidemia diinduksi dengan pemberian lemak babi murni dan kuning telur kepada tikus dua kali sehari dari hari ke-8 sampai hari ke-67. Penentuan kadar trigliserida dan kolesterol total dilakukan pada hari ke-0 dan hari ke-67. Temuan tersebut menunjukkan bahwa kelompok perlakuan dengan ekstrak etanol dari bayam merah pada dosis 200 mg/kgBB, 400 mg/kgBB, dan 800 mg/kgBB memiliki perbedaan yang signifikan secara statistik ($p < 0,05$) dibandingkan dengan kelompok kontrol negatif. Kelompok perlakuan III dengan dosis 800 mg/kgBB menunjukkan penurunan kadar kolesterol dan trigliserida paling banyak, sama dengan kelompok kontrol positif. Dapat disimpulkan bahwa ekstrak etanolik bayam merah terstandar memiliki aktivitas pencegahan terhadap aterosklerosis berdasarkan parameter kolesterol dan trigliserida total. Aktivitas tertinggi ditunjukkan oleh kelompok 800 mg/kgBB.

Kata kunci : Bayam merah; Hiperlipidemia; Kolesterol total; Terapi preventif; Trigliserida

INTRODUCTION

Hyperlipidemia is a condition in which the level of lipids in the plasma rises, including high levels of cholesterol and triglyceride.¹ Increased lipid levels in the blood may lead to atherosclerosis, a risk factor associated with cardiovascular diseases.² Cardiovascular diseases are the leading cause of death globally.³ To prevent high triglyceride and cholesterol levels, healthy lifestyle and good dietary habits are recommended. In addition, consumption of antihyperlipidemia drugs and traditional therapy using medicinal plants is believed to be able to reduce cholesterol levels. Herbal medicinal products and supplements have been massively used as the first-line treatment by approximately 80% of global population in the last three decades. The efficacy of some herbal products have been recognized, showing the great potential of medicinal plants in therapies; however, many others have yet to be tested or monitored.⁴ In fact, standardization is required to guarantee the quality of natural ingredients used in herbal products. Standardization ensures that the end products (drugs, extracts, or extract products) have constant parameters with guaranteed quality and safety. A standardized natural ingredient can be developed into products that benefit the society.⁵ In other words, a standardized natural ingredient has come closer to a possibly marketable herbal product. One of the diseases that can be empirically treated using such ingredient is hyperlipidemia.

Red spinach (*Amaranthus tricolor* L.) is one of the potential herbs for preventing increased levels of blood lipids. It contains a wide range of active ingredients, including vitamins, niacin, minerals (calcium, manganese, phosphorus, and iron), fibers, carotenoids, chlorophyll, alkaloids, flavonoids, saponins in the leaves, and polyphenols in the stems.⁶ There have been only few studies of the preventive actions of red spinach against

hyperlipidemia while it, in fact, has enormous potential for development since prevention is better than cure. This present study therefore aimed to examine the effects of administering a standardized ethanolic extract of red spinach (*Amaranthus tricolor* L.) as a preventive effort against increased total triglyceride and cholesterol to male Sprague-Dawley rats induced with high-lipid diet.

METHODS

Materials and equipment

This study used red spinach leaves, blue tip, 70% ethanol, mobile phase of methanol:ethanol (2:1), filter paper, cholesterol, pure lard, masker, microtube, LDL reagent, BR-II feed, gloves, silica gel F254, simvastatin, injection syringe, and oral syringe. The equipment included Pyrex glass, TLC chamber, Eppendorf tube, heating mantle (MTOPs, Gopal), rat observation cage, micropipette, capillary pipe, rotary evaporator (Heidolph with Heizbad WB type), centrifuge, analytical balance (Mettler Toledo with PL 303, Dragon 205 type), and material scale.

Research subjects

Inclusion criteria for the rats were Sprague-Dawley, male, healthy, aged 2-3 months, weighing 150-250 grams, obtained from one breeding place, and equally fed. The exclusion criteria would apply when the subjects were unhealthy during the adaptation phase.

Research procedure

This study has obtained an ethical clearance from the Health and Medical Research Ethics Committee of Islamic University of Indonesia (No. 88/Ka.Kom.Et/70/KE/III/2016).

Leaves of fresh red spinach were washed, aerated, dried, and milled to obtain powder. Weighing 20 grams in a filter paper bag, the powder was then put into a Soxhlet extractor along with the solvent (70% ethanol). Ethanol was used

as solvent due to its universality and remarkable capability of extracting quercetin. The ratio of powder to ethanol was 1:10, circulated seven times. A rotary evaporator was used to concentrate the extract residue at 60°C and 60 rpm. To obtain a paste-like texture, the viscous extract was evaporated on a water bath followed by weighing, packing in a glass container, and storing in a desiccator.⁷

Total cholesterol and triglyceride

Triglyceride level was examined using the Colorimetric Enzymatic Test “GPO” with spectrophotometry. This method works according to the principle of triglyceride measurement after an enzymatic breakdown by lipoproteinase. Chinonimine from the catalyzation of 4-aminoantipyrine by hydrogen peroxide became the indicator in this study. Meanwhile, the total cholesterol level in the serum was identified using the CHOD-PAP method with spectrophotometry and measured in mg/dl. Total cholesterol and

triglyceride levels were measured on day 0 and day 67. Grouping of experimental animals shown in Table 1.

Result analysis

The Kolmogorov-Smirnov test was used to identify the normality of total cholesterol and triglyceride data. Significance values higher than 0.05 indicate normally distributed data. In addition, the paired t-test statistically identified the effect of extract administration prior to and following the treatment.

RESULTS AND DISCUSSION

The results of standardization become a reference to establish that the ethanolic extract of red spinach has been standardized according to the standardization criteria based on the parameter values, which are presented in Table 2.⁸

Table 1. Grouping of experimental animals

No	Group	Protocols
1	Normal Control	Rats were fed standard BR-II and water ad libitum for 67 days.
2	Positive Control	Rats were induced by high-fat diet and duck yolk for 60 days since 8 th day. Two hours prior to induction, 0.09 mg/kgBW simvastatin was administered.
3	Negative Control	Rats were fed high-fat diet and duck yolk for 60 days starting from 8 th day.
4	Intervention 1	Rats were induced by high-fat diet and duck yolk for 60 days since 8 th day. A dose of 40 mg/200gBW ethanolic extract of red spinach was given two hours before induction (once a day).
5	Intervention 2	Rats were given high-fat diet and duck yolk for 60 days starting day 8. Administration of 80 mg/200gBW ethanolic extract of red spinach was done two hours prior to induction (once daily).
6	Intervention 3	Rats were fed high-fat diet and duck yolk for 60 days since day 8. Two hours prior to induction, 160 mg/200gBW ethanolic extract of red spinach was given (once a day).

Table 2. Standardization of ethanolic extract of red spinach leaves

No	Parameter	Result	Reference	Note
1.	Specific Parameter			
a.	Organoleptic	Odor: distinct spinach Taste: bitter Color: greenish black Texture: viscous	-	Suitable
b.	Yield	12.24%	-	Suitable
c.	Chromatographic pattern	R _f Value: 0.74-0.81	-	Suitable
d.	Quercetin content	29.29%	-	Suitable
e.	Water-soluble extractive value	24.38%	> 7%	Suitable
f.	Ethanol-soluble extractive value	12.06%	> 2.5%	Suitable
2.	Non-specific Parameter			
a.	Density	1.388 g/ml	> density of water (1 g/ml)	Suitable
b.	Moisture content	14.07%	5-30%	Suitable
c.	Acid-insoluble ash	0.51	1%	
d.	Microbial contamination			
1)	Yeast-fungal contamination	<10 ⁻² CFU/gram	< 10 ⁻² colonies/g	Suitable
2)	MPN coliform	Negative	< 5x10 ² colonies/g	Suitable
e.	Metal contamination			
1)	Pb	<0.096	< 10 mg/kg	Suitable
2)	Cu	16.70	< 50 ppm	Suitable

Hyperlipidemia preventive activity test

The antihyperlipidemia activity test for the ethanolic extract of red spinach leaves was conducted for 67 days. In the first 7 days, ethanolic extract of red spinach leaves was given without induction to the treatment groups I, II, III, and simvastatin was administered without induction to the positive control group. From day 8 to day 67, ethanolic extract of red spinach leaves and simvastatin were given with induced lard after 2 hours followed by oral induction of high-cholesterol diet (duck yolk) after 7 hours of extract administration. Sampling of blood serum was performed on day 0 and day 67. The rats were neither induced nor given a therapy, they were only fed with food and water ad libitum instead. Total cholesterol is one of the observed parameters to

identify hyperlipidemia. The difference in total cholesterol levels between pre-treatment and post-treatment is presented in Table 3.

The data in Table 3 shows that the negative control group had the highest increase in total cholesterol level compared to the other groups, reaching 66.29 mg/dl. Such condition indicates that the induction of high-fat diet was able to raise the cholesterol level. The greatest decrease in cholesterol level occurred to the positive control group (simvastatin) with 22.92 mg/dl reduction of total cholesterol level. Meanwhile, the administration of 800mg/kgBW of red spinach extract to the treatment group III could reduce 19.44 mg/dl cholesterol, marking it as the most significant decrease. Table 4 also shows the statistical

significance values obtained from the Paired Samples t-test to describe the significant differences before and after the treatment. The results indicate that only the normal group had insignificant difference between day 0 and day 67 ($p>0.05$) while the other groups experienced the opposite ($p<0.05$).

Triglyceride is also one of the parameters to identify a hyperlipidemia condition. The differences between pre-treatment and post-treatment are presented in Table 4, and the significance was analyzed using the Paired Sample t-test. Table 4 indicates that the negative group had the most significant increase in triglyceride level, reaching 89.02 mg/dl, compared to the other groups. The induction of high-fat diet consisting of lard and duck yolk could increase the triglyceride levels in rats. With -18.6 mg/dl decrease, the positive control group

administered with simvastatin became the group with the most significant triglyceride level reduction, followed by the treatment group III with -17.46 mg/dl reduction after given 800 mg/kgBW of extract and induced with high-fat diet. The statistical significance is also presented in Table 5 after an analysis using the Paired Sample T-Test. The normal group and the treatment group II had insignificant differences between day 0 and day 67 ($p>0.05$), but the other groups experienced the opposite ($p<0.05$). Administration of high fat and egg yolk leads to increased total cholesterol and triglyceride levels. The decrease in total cholesterol and triglyceride levels depends on the doses of the extract; the higher the dose, the higher potential it has in preventing hyperlipidemia.

Table 3. Mean of total cholesterol levels on day 0 and 67

Groups	Mean of Total Cholesterol Levels			
	Day 0	Day 67	Δ	Sig.
Normal	72.94 \pm 4.88	69.18 \pm 6.14	-3.76	0.089
Negative	80.78 \pm 6.93	147.07 \pm 7.60	66.29	0.000*
Positive	78.72 \pm 6.39	55.80 \pm 6.22	-22.92	0.001*
Treatment I (P1)	73.72 \pm 5.95	80.64 \pm 5.29	-6.92	0.003*
Treatment II (P2)	69.98 \pm 4.77	66.02 \pm 6.24	-3.96	0.006*
Treatment III(P3)	69.56 \pm 5.93	50.12 \pm 8.40	-19.44	0.002*

Note : All the means of cholesterol levels in rats are in mg/dl

Δ : Difference in mean cholesterol levels in rats between day 0 and day 67

* : Statistically significant difference between day 0 and day 67

Table 4. Mean of triglyceride levels \pm SD before and after the therapy and induction of high-fat diet (day 0 and day 67)

Group	Mean of Total Triglyceride Levels			
	Day 0	Day 67	Δ	Sig.
Normal	62.12 \pm 7.64	60.76 \pm 6.57	-1.36	0.439
Negative	63.94 \pm 7.84	152.96 \pm 7.81	89.02	0.000*
Positive	64.78 \pm 5.2	46.18 \pm 2.81	-18.6	0.004*
Treatment I (P1)	59.9 \pm 6.15	69,06 \pm 8.27	9.16	0.019*
Treatment II (P2)	64.58 \pm 4.86	63.02 \pm 13.23	-1.56	0.755
Treatment III (P3)	58.5 \pm 4.92	41.04 \pm 4.55	-17.46	0.002*

Note : All the means of triglyceride levels in rats are in mg/dl

Δ : Difference in mean triglyceride levels in rats between day 0 and day 67

* : Statistically significant difference between day 0 and day 67

The mechanism of action of quercetin in reducing total cholesterol levels includes its ability to inhibit the secretion of apo B-100 in CaCO₂ cells and to reduce the activity of MTP in forming cholesterols. In addition, quercetin is capable of restraining the activity of HMG-KoA reductase, an enzyme playing a role in cholesterol production, and quercetin can function as an antioxidant to combat free radicals.^{9,10} Furthermore quercetin can function as antioxidants that suppress the release of reactive O₂ thus reducing endothelial dysfunction by inhibiting the initiation of oxidative chain reactions and therefore preventing the production of more macrophages. Antioxidants also decrease the toxicity of oxidative LDL against endothelial cells as well as reduce oxidative degradation due to nitric oxide.^{11,12}

CONCLUSION

The standardized ethanolic extract of red spinach (*Amaranthus tricolor* L.) has the potential as a preventive therapy for atherosclerosis based on the total cholesterol and triglyceride parameters in male Sprague-Dawley rats. Higher extract dose leads to higher potential to prevent increased total cholesterol and triglyceride levels. This study found that the optimum dose of ethanolic extract of red spinach leaves was 800 mg/kgBW in the treatment group III.

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