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Effects of ethyl acetate extracts of *Marsilea crenata* Presl. leaves on risks of atherosclerosis in dyslipidemic rats

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ABSTRACT

Background: Dyslipidemia is widely known as a significant contributor to the incidence of cardiovascular diseases (CVD). Over the years, traditional therapeutic strategies, including the use of herbs, have been recognized. *Marsilea crenata* Presl. (MC), or a plant known for its phytoestrogen content, has shown biological activities similar to oestrogen. These activities comprise the reduction of serum cholesterol levels, thereby having potential roles in managing dyslipidaemia and its associated cardiovascular risks.

Objective: This study aims to examine the effects of ethyl acetate extracts of MC leaves on the serum lipid profile of experimental rats with dyslipidaemia induced by high-fat diet (HFD).

Methods: This study applied a pretest-post-test study design with control group, involving 30 rats which are divided into 5 treatment groups, namely N (normal), HFD, STA (Simvastatin), MC_1, MC_2, and MC_3. Group N was only treated with standard feed, and the others received HFD on days 1-14. On the 15^{th} – 28^{th} day, the HFD group was treated with standard feed, and the STA group received simvastatin 0.36 mg/200 g BW/day. The groups of MC_1, MC_2, and MC_3 were treated with oral MC extracts at 100, 200, and 400 mg/kgBW, respectively. Their body weight was measured at the beginning of the study, as well as after 2 and 4 weeks. Subsequently, the experimental rats were fasted overnight on the 14^{th} and 28^{th} day. Their blood was collected on the 15^{th} day (pre-test) and 29^{th} day (post-test) to measure serum concentration of total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-c). Low-density lipoprotein cholesterol (LDL-c) concentration was also calculated by using the Friedewald formula.

Results: MC_3 extracts significantly reduced the TC (-40.95 ± 1.77), TG (-46.12 ± 3.79), and LDL-c levels (-0.017 ± 0.010), and it also increased levels of HDL-c (11.28 ± 2.34) mg/dL in the HFD-induced rats.

Conclusion: Ethyl acetate extracts of MC leaves could reduce risk factors for atherosclerosis by returning lipid profile of the dyslipidemic rats to normal conditions with an increased dose.

***Latar belakang**: Dislipidemia dikenal luas sebagai kontributor signifikan terhadap kejadian penyakit kardiovaskular (CVD). Selama bertahun-tahun, strategi terapi tradisional termasuk penggunaan herbal telah mendapat pengakuan. Marsilea crenata, Presl (MC), tanaman yang terkenal dengan kandungan fitoestrogennya, telah menunjukkan aktivitas biologis yang mirip dengan estrogen. Kegiatan-kegiatan ini terdiri dari pengurangan kadar kolesterol serum, sehingga menunjukkan potensi peran dalam mengelola dislipidemia dan risiko kardiovaskular terkait.*

***Tujuan:** Untuk menguji pengaruh ekstrak etil asetat daun MC terhadap profil lipid serum tikus percobaan penderita dislipidemia yang diinduksi diet tinggi lemak (DTL).*

***Metode:** Desain penelitian pretest-posttest dengan kontrol menggunakan 30 ekor tikus yang dibagi menjadi*

5 kelompok perlakuan yaitu N (normal), DTL, Simvastatin (STA), MC1, MC2, dan MC3. Kelompok N hanya diberi pakan standar dan kelompok lainnya mendapat DTL pada hari ke-1 hingga ke-14. Pada hari ke 15 hingga 28, kelompok DTL diberi pakan standar, dan STA mendapat simvastatin 0,36 mg/200 g BB/ hari. Kelompok MC1, MC2, dan MC3 diberi ekstrak MC oral masing-masing 100, 200, dan 400 mg/kg BB. Berat badan diukur pada awal penelitian, serta setelah 2 dan 4 minggu. Setelah tikus percobaan dipuasakan semalam, darah dikumpulkan pada hari ke-15 (pre-test) dan ke-29 (post-test) untuk mengukur konsentrasi serum kolesterol total, trigliserida, dan kolesterol lipoprotein densitas tinggi. Konsentrasi kolesterol lipoprotein densitas rendah dihitung menggunakan rumus Friedewald.

Hasil: *Ekstrak MC3 secara signifikan menurunkan kadar kolesterol total (-40,95 ± 1,77), trigliserida (-46,12 ± 3,79), dan kolesterol lipoprotein densitas rendah (-0,017 ± 0,010), serta meningkatkan kadar kolesterol lipoprotein densitas tinggi (11,28 ± 2,34) mg/dL pada tikus yang diinduksi DTL.*

Kesimpulan: *Ekstrak etil asetat daun MC menurunkan faktor risiko aterosklerosis dengan mengembalikan profil lipid tikus dislipidemia ke kondisi normal dengan peningkatan dosis.*

INTRODUCTION

Dyslipidemia or hypercholesterolemia is the imbalance of lipid characterized by higher concentration of TC, TG, and LDL-c, or a decreased level of HDL-c, compared to normal condition.[1] This condition is related to the onset of cardiovascular disease (CVD) such as atherosclerosis, myocardial infarction, and cerebral vascular accidents (CVA), which are the main causes of global burden disease.[2]

The modern pharmacological therapy for abnormal lipid is effective but expensive with adverse drug reactions causing patients to be non-adherent in taking medication. Furthermore, it has been reported that herbal remedies can cause a few side effects, such as fatigue.[3] The use of nutraceuticals containing monomers and herbal derivatives has been proven to be safe and well tolerated, compared to statins.[4] These compounds have been recommended as an alternative to lowering lipid in statin-intolerant patients.[5]

In a previous in vitro study, the methanol extracts of water clover or MC leaves produced higher inhibitory effect on the Hydroxymethylglutaryl-CoA (HMG-CoA) enzyme. Dried clover leaf extracts with a solvent ratio of ethyl acetate-methanol (100:0) have a total flavonoid content of 29.47 ± 0.30 mg QE/g extract, antioxidant activity (70.19 mg/L), and inhibited HMG CoA by 95.12%.[6,7]

Dietary intake of six flavonoid classes (flavonols, anthocyanidins, proanthocyanidins, flavones, flavanones, and flavan-3-ols) can reduce risks of CVD. Greater intake is associated with reduced risks of CVD,[8] and phytoestrogen therapy is a promising adjuvant to reduce the risks.[9] This study aimed to investigate the biological activities of ethyl acetate extracts of MC leaves on the lipid profile, which is a significant risk factor for atherosclerosis in rat model of high-fat diet-induced dyslipidemia.

METHODS

Study design

This study applied pretest-post-test design with control group, involving 30 rats divided into 6 treatment groups, namely N (normal), HFD, STA, MC_1, MC_2, and MC_3. Group N (5 rats) was only treated with standard food, while others (25 rats) were treated with standard food and 2 mL HFD twice a day orally for 28 days to obtain dyslipidemic rats. From the 15^{th} to 28^{th} day, the rats in the HFD group served as the negative control. The STA group, representing the positive control group, received 0.18 mg/ml/200 mgBW of simvastatin. Meanwhile, the MC groups received varied doses of 100, 200, and 400 mg/kgBW of the MC extracts, respectively. The rats' serum lipid profile was analysed on the 15th day (as a pre-test data) and on 29^{th} day (as a post-test data).

Tools and materials

The tools used in this study were cages, spectrophotometer, scales, glasses, centrifuge, oral gavage feeding needle, micropipette, syringe, etc. The rats were male rats, *Rattus norvegicus domestica* (albino), with weight of 180-200 grams, with age of 2-3 months, and in healthy condition. This study also utilized total cholesterol assay kit (DIASYS), triacylglycerol assay kit (DIASYS), ethyl acetate p.a. (pro-analysis, e-Merck), BR-2, CMC-Na 0.1%, aquadest, and simvastatin 10 mg tablet.

Extraction

The water clover or MC plants were obtained in the rice fields of the Gogik Ungaran area and examined at the Faculty of Science and Mathematics, Universitas Diponegoro. The

leaves of MC plants were washed thoroughly with running water, drained, and aired in a place not exposed to direct sunlight until drying. Subsequently, grinding was performed to obtain a fine powder and sifted with a 30/40 mesh sieve. A total of five hundred grams of MC leaf powder was macerated and remacerated in 3.250 ml and 1.750 ml of ethyl acetate for 3 x 24 hours and 2 days. The filtrates (extracts) were collected in a container and evaporated at 77 °C temperature with a rotary evaporator.

Animal feed

The rats were fed with BR 2 Comfeed consisting of water (12%), protein (19%), crude fat (4%), crude fibre (4.5%), calcium (0.9-1.1%), phosphorus (0.7%), and a combination of coccidiostat + antibiotics (0.9%) as a standard feed. The composition of HFD was beef tallow (10%), used cooking oil (20%), and quail egg yolk (20%) mixed with 120 ml of water.

Ethical clearance

This study was approved by the Medical Research Ethics Committee of the Faculty of Medicine, Universitas Islam Sultan Agung, Semarang, with Ref. No. 361/XI/2020.

Animal preparation

The 2–3-month-old male rats, with body weight of 180–200 g, were obtained from Pasar Hewan Depok (a pet market), Surakarta. The animals' conditions were checked by the Department of Agriculture, Food Security and Fisheries, Surakarta City Government, and were considered healthy with a certificate No. 524.3/510.M/SKKH). Before the experiment, the rats were acclimatized in the Pharmacology Laboratory of Universitas Ngudi Waluyo at room temperature 23-27°C, relative humidity 70 – 90%, and lighting for 12 hours (lights on from 18.00 - 06.00) for 7 days. They were fed with BR-2 ad libitum and had free access to drinking water.

Profile lipid analysis

The experimental animals were weighed on the 1st day (baseline), 15th day (pre-test), and 29th day (post-test), and fasted overnight on the 14th and 28th day. Their blood samples were taken from the lateral vein on the 15th and 29th day. Furthermore, TC, TG, and HDL-c levels were measured spectrophotometrically. A total of 200 µL of blood was put into a centrifuge tube, left for 10-20 min, and centrifuged at 3000 rpm for 15 minutes. The serum was separated by putting 10 µL into a serology tube. A 10,000 µL assay kit was added and incubated at 37°C for 10 minutes. Absorbance was read at a maximum wavelength (λmax) of 500 nm, and concentration of TC, TG, HDL-c, and LDL-C was calculated with the formula in the following.

$$\text{Total cholesterol } (\frac{mg}{dL}) = \frac{\text{A sample}}{\text{A standard}} \text{ x C standard...(1)}$$

$$\text{Triglyceride } (\frac{mg}{dL}) = \frac{\text{A sample}}{\text{A standard}} \text{ x C standard...(2)}$$

$$\text{HDL-c } (\frac{mg}{dL}) = \frac{\text{A sample}}{\text{A standard}} \text{ x C standard ...(3)}$$

$$\text{LDL-c } (\frac{mg}{dL}) = \text{TC-HDL-c - } (\frac{TG}{5}) \text{ (Friedewald formula)...(4)}$$ [10]

Statistical analysis

The t-test and one-way ANOVA were performed, followed by the Least Significant Difference (LSD) analysis to investigate differences in TC, TG, HDL-c, and LDL-c levels by using SPSS version 20.0 software. A p-value <0.05 was declared as a statistically significant difference.

RESULTS

The highest weight loss occurred in the MC_3 group, followed by groups of MC_2, MC_1, and STA; meanwhile the lowest was in the HFD group. The ANOVA analysis demonstrated a significant difference in ΔBW among the groups. The ΔBW values in the MC_2 and MC_3 were different from the HFD group, unlike the MC_1. Treatments with MC were able to reduce the body weight of the rats, and the MC_3 had a weight-loss effect (Table 1).

Table 2 explained biological activities of MC extracts on TC levels (ΔTC). The lowest ΔTC was in the MC3 group, followed by groups of STA, MC2, MC1, and HFD. The one-way ANOVA showed significant differences in ΔTC among the study groups. The decrease in TC levels (ΔTC) in the MC1, MC2, and MC3 groups was significantly different from the HFD group. Oral administration of the MC leaf ethyl acetate extracts could reduce the TC levels. An MC3 treated to the dyslipidemic rats could reduce the TC levels (-40.04 ± 1.77 mg/dL), and its effectiveness was no different with simvastatin.

Table 3 demonstrated the biological activities of MC in reducing the TG levels. The highest decrease

Table 1. The effects of *Marsilea crenata* on body weight in the dyslipidemic rats

Groups	Body weight (gram)				$p_{t\text{-test}}$
	Baseline	Pre-test	Post-test	ΔBW	
N	189.52 ± 1.32	188.50 ± 4.64	198.17 ± 9.67	9.67 ± 4.84	>0.05
HFD	190.40 ± 1.30	230.67 ± 3.56	227.33 ± 1.03	-3.34 ± 4.32	>0.05
STA	189.57 ± 1.73	211.80 ± 2.17	205.60 ± 1.14	-6.20 ± 3.19	<0.05
MC_1	189.04 ± 1.61	232.50 ± 2.88	225.83 ± 0.98	-6.67 ± 2.34	<0.05
MC_2	188.92 ± 3.01	231.33 ± 2.42	213.33 ± 2.16	-18.00 ± 3.16	<0.05
MC_3	188.50 ± 2.06	233.17 ± 3.25	203.17 ± 1.47	-30.00 ± 3.35	<0.05
p_{Anova}	>0.05	<0.001*	<0.001*	<0.001*	

N: Normal; HFD: High Fat Diet; STA: Simvastatin; MC_1: *Marsilea crenata* 100 mg/kgBW; MC_2: *Marsilea crenata* 200 mg/kgBW; MC_3: *Marsilea crenata* 400 mg/kgBW; ΔBW: body weight difference, p<0.05

Table 2. Biological activities of *Marsilea crenata* on TC levels in the dyslipidemic rats

Groups	Body weight (gram)			$p_{t\text{-test}}$
	Pre-test	Post-test	Δ TC	
N	77.57 ± 1,71	78.08 ± 1.30	0.51 ± 2.27	>0.05
HFD	145.97 ± 1,14	136.06 ± 1.67	-9.91 ± 1.40	<0.05
STA	146.93 ± 2,26	105.75 ± 1.49	-41.18 ± 2.17	<0.05
MC_1	147.88 ± 1,59	129.97 ± 1.20	-17.91 ± 2.03	<0.05
MC_2	147.15 ± 2,18	121.83 ± 1.69	-25.32 ± 2.44	<0.05
MC_3	147.51 ± 1,69	106.56 ± 1.33	-40.95 ± 1.77	<0.05
p_{Anova}	>0.05	<0.001*	<0.001*	

TC: total cholesterol N: Normal; HFD: High Fat Diet; STA: Simvastatin; MC_1: *Marsilea crenata* 100 mg/kg BW; MC_2: *Marsilea crenata* 200 mg/kg BW; MC_3: *Marsilea crenata* 400 mg/kg BW; ΔTC: total cholesterol difference, p<0.05

in TG was in the STA group, followed by groups of MC_3, MC_2, and MC_1; meanwhile, the lowest was in the HFD group. The results of ANOVA analysis revealed significant differences among the groups. The ΔTG in the MC_1, MC_2, and MC_3 groups were significantly different from the HFD group. Oral administration of MC leaf ethyl acetate extracts could reduce the TG levels. Similarly, MC_3 treated to the dyslipidemic rats could reduce the TG levels (-46.12 ± 3.79 mg/dL), and its effectiveness was not different from simvastatin.

Table 4 portrayed the highest increase in HDL-c occurred in the STA group, followed by groups of MC_3, MC_2, and MC_1; meanwhile, the lowest was in

Table 3. Biological activities of MC extracts on TG levels in the dyslipidemic rats

Groups	TG levels (mg/dL)			$p_{t\text{-test}}$
	Pre-test	Post-test	Δ TC	
N	91.85 ± 2.88	89.21 ± 1.66	-2.64 ± 1.90	>0.05
HFD	165.56 ± 2.58	154.13 ± 1.20	-11.43 ± 2.04	<0.05
STA	164.85 ± 1.74	118.13 ± 1.41	-46.72 ± 2.95	<0.05
MC_1	165.06 ± 2.27	140.06 ± 1.16	-25.02 ± 3.36	<0.05
MC_2	167.92 ± 4.24	131.71 ± 2.33	-36.21 ± 5.16	<0.05
MC_3	165.56 ± 2.28	119.44 ± 2.07	-46.12 ± 3.79	<0.05
p_{Anova}	<0.001*	<0.001*	0.001*	

TG: triglyceride; N: Normal; HFD: High Fat Diet; STA: Simvastatin; MC_1: *Marsilea crenata* 100 mg/kg BW; MC_2: *Marsilea crenata* 200 mg/kg BW; MC_3: *Marsilea crenata* 400 mg/kg BW; ΔTG: triacylglycerol difference, p<0.05

the HFD group. Based on the analysis of variance, HDL-c levels among the groups were significantly different. ΔHDL-c levels in MC_1, MC_2, and MC_3 groups were different from the HFD group. Oral administration of MC leaf ethyl acetate extracts could increase the HDL-c levels. Furthermore, MC_3 given to the dyslipidemic rats could increase the HDL-c levels (11.28 ± 2.34 mg/dL), and its effectiveness was not significantly different from simvastatin.

Table 5 illustrated the highest decrease in LDL-c occurred in the MC_3 group, followed by groups of MC_2, STA, and MC_1; meanwhile, the lowest was in the HFD group. Based on one-way ANOVA, the LDL-c levels were significantly different from other groups. ΔLDL-c in MC_1, MC_2, and MC_3 group was significantly different from the HFD group. Oral administration of MC leaf ethyl acetate extracts could decrease the LDL-c levels. Then MC_3 treated to the dyslipidemic rats could reduce the LDL-C levels (- 0.017 ± 0.010 mg/dL), and it was as effective as simvastatin.

DISCUSSION

Based on this study, the HFD induction for 2 weeks caused a significant increase in the body

Table 4. Biological activities of MC extracts on HDL-c levels in the dyslipidemic rats

Groups	HDL-c levels (mg/dL)			$p_{t\text{-test}}$
	Pre-test	Post-test	ΔHDL-c	
N	84.23 ± 0,99	78.20 ± 13.30	-6.23 ± 1.07	>0.05
HFD	55.94 ± 3,67	57.38 ± 1.87	1.44 ± 5.30	>0.05
STA	60.70 ± 2,47	73.92 ± 0.93	13.22 ± 2.07	>0.05
MC_1	57.30 ± 1,93	63.60 ± 0.66	6.30 ± 2.16	<0.05
MC_2	58.20 ± 5,91	65.10 ± 4.55	6.90 ± 5.99	>0.05
MC_3	60.00 ± 2,30	71.28 ± 0.39	11.28 ± 2.34	<0.05
p_{Anova}	<0.001*	<0.001*	0.001*	

HDL-c: High-Density Lipoprotein cholesterol; N: Normal; HFD: High Fat Diet; STA: Simvastatin; MC_1: *Marsilea crenata* 100 mg/kg BW; MC_2: *Marsilea crenata* 200 mg/kg BW; MC_3: *Marsilea crenata* 400 mg/kg BW; ΔHDL: High-Density Lipoprotein cholesterol difference, *:p<0.05

Table 5. Biological activities of MC extracts on LDL-c levels in the dyslipidemic rats

Groups	LDL-c levels (mg/dL)			$p_{t\text{-test}}$
	Pre-test	Post-test	ΔLDL-c	
N	0.17 ± 0.01	0.17 ± 0.00	-0.00 ± 0.01	>0.05
HFD	0.08 ± 0.02	0.06 ± 0.00	-0.03 ± 0.02	<0.05
STA	0.07 ± 0.01	0.07 ± 0.01	-0.00 ± 0.02	>0.05
MC_1	0.08 ± 0.02	0.03 ± 0.01	-0.06 ± 0.01	<0.05
MC_2	0.08 ± 0.02	0.02 ± 0.04	-0.07 ± 0.07	<0.05
MC_3	0.07 ± 0.01	0.05 ± 0.01	-0.02 ± 0.01	<0.05
p_{Anova}	0.015*	<0.001*	<0.001*	

LDL: Low-Density Lipoprotein cholesterol; N: Normal; HFD: High Fat Diet; STA: Simvastatin; MC_1: *Marsilea crenata* 100 mg/kg BW; MC_2: *Marsilea crenata* 200 mg/kg BW; MC_3: *Marsilea crenata* 400 mg/kg BW; ΔLDL: Low-Density Lipoprotein cholesterol difference, *:p<0.05

weight and hypercholesterolemia. In another study, HFD was used as an inducer to obtain obese rats.[11] The induction is also used to initiate hypercholesterolemia in studying cholesterol metabolism in vivo.[12,13] Hypercholesterolemia leads to the development of metabolic syndrome such as atherosclerosis, diabetes mellitus, hypertension,[14] lipid metabolism disorders, chronic renal failure, nephrotic syndrome, and hypothyroidism.[15] Induction of HFD results in significant obesity in rats characterized by weight gain, hemodynamic changes, increased levels of serum lipid, insulin,

leptin, Apolipoprotein (Apo)-B, and decreased Apo-A1 and HDL-c, Na/K pump (Naþ/Kþ), cardiac serum adenosine triphosphate (ATP)-ase activity, and antioxidant levels.[16]

Based on this study, the weight of rats was significantly reduced at medium (MC_2) and high (MC_3) doses. Similarly, treatment with *Camellia japonica* fruit (CJF) medium dose (400 mg/kgBW), and high dose (800 mg/kgBW) could reduce the body weight to a greater extent than the control group.[14] Weight loss was due to the consumption of MC-containing tannin, which regulated glycolipid metabolism, improved insulin resistance, and reduced hepatic oxidative stress (OS). The MC extracts have anti-obesity effect, possibly through the regulation of adipogenesis, and treatment with the MC for 2 weeks significantly could restore cholesterol metabolism to normal conditions and could maintain lipid profile comparable to the normal group; meanwhile, treatment with CJF took 1 month.[14]

Camellia japonica (Family: Theaceae) and *M. crenata* (Family: Marsileaceae) are aquatic plants belonging to the hydrophyte group, which thrive in rice fields, ponds, lakes, swamps, and rivers. The CJF is able to have anti-atherogenic activity mediated by decreased serum TC and TG and increased HDL, inhibit lipid accumulation in the inner vessel wall, and significantly reduce peroxidation in rats induced by HFD.[14]

This study proves that the biological activities of the MC extracts can significantly reduce the TC levels in the dyslipidemic rats. The efficacy of MC_3 is as effective as simvastatin, and the results regarding the anti-atherosclerotic effects of the MC may add to the evidence that lowering cholesterol levels may be beneficial and necessary to reduce CVD risks. These results confirm the study of Jeong et al. revealing that increased cholesterol levels is associated with high risks of CVD.[17]

Lowering cholesterol levels is associated with reducing risks of ischemic heart diseases (IHD). Furthermore, a reduced risk of cerebrovascular disease (CBVD) was observed in high-low cholesterol compared with the sustained high group.[17] That study may add to the evidence that MC extracts have beneficial anti-atherosclerosis biological activities and are necessary for those diagnosed with dyslipidaemia to reduce CVD risks.

Hypertriglyceridemia is often accompanied by lipoprotein disturbances, including an increase in VLDL, total apolipoprotein (apo) C-III, and LDL-c particles, as well as decreased levels of HDL-c associated with increased levels of CVD risks.[18] Plasma TG levels are known to correspond to levels of TG-rich lipoproteins (TRLs).[19] The TRL and specific markers of TG metabolism, such as lipoprotein lipase (LPL) and Apolipoprotein C-III (apo C-III), contribute to the development of atherosclerosis and CVD both directly and indirectly.[17] However, there is inconsistent evidence that triglycerides are on the pathway to cause atherosclerosis from cardiovascular outcome studies regarding the reduction of cardiovascular risks by triglyceride-lowering agents.[20] This means that there is still some uncertainty regarding the direct causal role of TRL in the development of atherosclerosis and CVD.[21]

Marsilea crenata treatments were able to reduce TG levels in the HFD-induced rats. Administration of MC_3 could reduce the TG levels as effectively as simvastatin in the HFD-induced dyslipidemic rats, preventing an increased risk of CVD. These results complement plants proven to be TG-lowering agents with the right mechanism of action, such as *Allium sativum*, *Nigella sativa*, *Curcuma longa*, *Anethum graveolens*, and *Commiphora muku*l.[22]

This study indicated that MC_3 treatment could increase the HDL-c levels 9,84 mg/dL in the dyslipidemic rats. At this dose, its effectiveness was not different from administering the gold standard drug (simvastatin). The same results were revealed by a study of Lee et al. demonstrating that CJF (800 mg/kg BW) significantly could increase the HDL-c levels 1.8 fold.[14] High-density lipoprotein cholesterol shows several physiological activities having important roles in reducing risks of atherosclerosis, beneficial activities on platelet function, endothelial function, coagulation parameters, inflammation, and interactions with triglyceride-rich lipoproteins. Clinical and experimental data points out that HDL-c has antioxidant effect, significantly contributing to protection from atherosclerosis.[23]

The main anti-atherosclerotic activity of HDL-c is through reverse cholesterol transport. HDL scavenges cholesterol from the peripheral blood by transportation to the liver and is excreted in the biliary system. This compound has been shown to possess beneficial effects on platelet and endothelial function, coagulation parameters, inflammation, and interactions with triglyceride-

rich lipoproteins.[23] Therefore, HDL can reduce the process of atherosclerosis.[24]

The liver controls circulating lipoprotein concentration primarily through its service using LDL receptors on the surface of the liver.[25] The guidelines on dyslipidemia advocate lowering LDL-c to treatment goals based on an individual's CVD risks.[26]

Oxidized LDL (ox-LDL) can trigger the expression of adhesion molecules on the cell surface, stimulating the activation of endothelial cells (EC).[27] These molecules mediate the rolling and adhesion of blood leukocytes, adhere to the endothelium in response to chemokines (and chemotactic cytokines), and migrate to intima. Therefore, macrophage activation releases proinflammatory cytokines, ROS synthesis, as well as the production of proteolytic enzymes and causes metric degradation. This causes plaque destabilization.[28]

Oxidized LDL also binds to lectin-like ox-LDL receptor-1 (LOX-1).[29] The key molecule in the pathogenesis of atherosclerosis is LOX-1, and the inhibition demonstrates significant changes.[29]

The LOX-1 consists of a short N-terminal cytoplasmic domain, a transmembrane, a neck region, and an extracellular C-type lectin-like domain (CTLD), forming a type II integral membrane glycoprotein, which is described as an important ox-LDL receptor on EC cells.[30] The expression in normal conditions is very low when pro-atherogenic or pro-inflammatory trigger responses to increase. The LOX-1 levels are upregulated in the early stages of atherogenesis in ECs and plaques at later stages. In addition, intimal smooth muscle cells (SMCs) and macrophages demonstrate increased expression in human carotid atherosclerotic plaque.[31] The LOX-1 may also play an important role in the inflammatory response and lipid deposition in mouse heart vessels.[32]

Atherosclerosis is a disease resulting from the accumulation of lipid peroxidation and inflammatory cells in the arterial walls caused by an imbalance between the formation of reactive oxygen species (ROS) and the antioxidant defence system. These increase OS and contribute to endothelial dysfunction. Increased ROS causes oxidation of native LDL to ox-LDL, which plays an important or determining role in atherogenesis.[31]

The anti-atherosclerotic activity of MC extracts is due to the content of flavonoid compounds which act as antioxidants, counteract ROS, and inhibit OS to prevent the formation of ox-LDL and atherosclerosis. This was confirmed by other studies which identified the presence of flavonoids and steroid compounds.[33] Flavonoids are known for their anti-inflammatory, antihypertensive, vasodilator, anti-obesity, anti-hypercholesterolemic, and anti-atherosclerotic effects.[34]

This study proves that treatment with MC_3 extracts can prevent risks of atherosclerosis by reducing LDL-c levels. This effect is caused by the biological activities of the flavonoid content of the MC extracts by reducing OS, inhibiting ox-LDL and platelet aggregation, and acting as a vasodilator in blood vessels.[35] Limitations in this study include the lack of control over the HFD consumption, absence of checks on diabetes status, and insufficient monitoring of the physical activities of the rat known to be risk factors for dyslipidaemia.

CONCLUSION

In conclusion, oral administration of the MC leaf ethyl acetate extracts prove to reduce atherosclerosis risk factors by returning the TC, TG, HDL-c, and LDL-c levels to normal conditions in the HFD-induced dyslipidaemia rats. The MC_3 (400 mg/kg BW) have anti-dyslipidaemia activities to prevent risk of atherosclerosis which is not significantly different from the activities of the gold standard simvastatin. Based on this study, the MC extract is a potential herb to reduce atherosclerosis risk factors. Further studies are necessary to prove the safety and efficacy of the MC in clinical conditions.

CONFLICT OF INTEREST

The authors declare that they have no competing interests or conflicts that may have influenced the study outcome.

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AUTHOR CONTRIBUTION

HDL and LDL results and analysis were

conducted by FNA, while the TC and TG conducted by ANF who is supervised by JS and DO. The results were discussed together guided by the research team leader. All members played active roles in studying and preparing this manuscript.

LIST OF ABBREVIATION

Apo C-III: apolipoprotein C-III; Apo-A: apolipoprotein-A; Apo-B: apolipoprotein-B; ATP: adenosine triphosphate; BW: body weight; CBVD: cerebrovascular disease; CJF: Camellia japonica fruit; CTLD: C-type lectin-like extracellular domain; CVA: cerebral vascular accidents; CVD: cardiovascular disease; EC: endothelial cell; HDL-c: high-density lipoprotein cholesterol; HFD: high fat feed; HMG-CoA: hydroxymethylglutaryl-Coenzyme A; IHD: ischemic heart disease; ILEP: International Lipid Expert Panel; LDL-c: low-density lipoprotein cholesterol; LOX1: lectin-like oxidized low-density lipoprotein receptor-1; LPL: lipoprotein lipase; MC: Marsilea crenata. ox-LDL: oxidized low-density lipoprotein; p.a.: pro analysis; QE: quercetin equivalent; ROS: reactive oxygen species; SMCs: smooth muscle cells; TC: total cholesterol; TG: triglycerides; TRLs: triglyceride-rich lipoproteins

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