



Antiatherogenic effect of *Mezzetia parviflora* BECC. extract in high cholesterol fed rats

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ABSTRACT

The investigation of anti-atherosclerotic activity of *M. parviflora* and the mechanisms responsible for the extract's effects (antihypolipidemic, anti-lipidperoxidation, and monocyte chemoattractant protein-1 (MCP-1) inhibition activities) has been performed. In this study, atherogenesis was induced by feeding rats with a hypercholesterolemic diet (200 mg/Kg day⁻¹) for 3 months. The cholesterol intake was administered along with simvastatin 3.6 mg/Kg or *M. parviflora* extract at doses of 100, 300 and 500 mg/Kg. Atheroma observation was performed by immunohistochemistry staining method with anti-macrophage antibody (MAC387). Animal plasma biochemical (total cholesterol, triglyceride, HDL, LDL, malondialdehyde, and MCP-1) profiles were measured. *M. parviflora* extract dose dependently decreased the total-cholesterol, triglyceride, and LDL-cholesterol levels; and increased HDL-cholesterol in atherogenic-diet rats. Furthermore, *M. parviflora* extract lowered the lipid-peroxidation (malondialdehyde, MDA) and MCP-1 production in dose dependent manner. Immunohistochemistry staining with anti-macrophage antibody (MAC387) showed that atherogenic-diet rats treated with *M. parviflora* extract at a dose of 500 mg/Kg was not colored positively, the same as that in normal and simvastatin treated groups. These results suggest that *M. parviflora*-standardized extract may have anti-atherosclerosis effect through antihyperlipidemia and antilipid-peroxidation activities.

Keywords: *Mezzetia parviflora* Becc., Antiatherosclerosis, Antioxidant, Anticholesterol, MCP-1

INTRODUCTION

Degenerative diseases such as coronary heart, stroke, and diabetes are deadly diseases and have become problems in both developed and developing countries. The result of Indonesia Basic Health Research in 2007 revealed that the prevalence of cardiovascular disease and stroke in Indonesia were 7.2% and 0.83 %, respectively [1]. The main process responsible for the occurrence of coronary heart and stroke diseases is atherosclerosis [2].

Atherosclerosis is the result of a continual inflammatory response. Inflammation is involved in all stages of atherosclerosis from initiation, progression, to complication of thrombosis [3, 4, 5]. Some factors that trigger inflammatory responses can be found in our internal and external environments such as excessive consumption of hydrogenated oil; obesity; smoking; exposure to radiation; toxin from environment; problems due to free radicals; bacterial and virus infections; emotional stress; and even some medicines [6].

Studies on the pathogenesis of atherosclerosis show that the alterations in endothelial-cell function precede the development of atherosclerotic changes and the progression of vascular lesions. Endothelial dysfunction occurs well before the structural manifestation of atherosclerosis and becomes an independent predictor of future cardiovascular events [7]. It is a result of a continual process of endothelium layer stimulation and activation that can be caused by imbalanced production between oxidant and antioxidant in human body. It leads various occurrences such as LDL oxidation and inflammation response which worsens the condition [8].

Oxidized LDL (OxLDL) produce some biological responses, firstly it induces the endothelial cell to generate some chemotactic substances including *monocyte chemotactic protein-1* (MCP-1). MCP-1 attracts circulating monocytes, and promotes the differentiation of resident-monocytes into tissue macrophages and inhibits the motility of resident macrophages. These processes increase foam cell formation and finally cause atherosclerosis [9-12].

The prevention of LDL oxidation is not yet widely known. The first strategy to prevent the LDL oxidation in antiatherosclerotic research is mechanism-based antioxidant therapy [3]. One of the sources of natural antioxidants is herbal medicine with high content of polyphenol compounds. The consumption of phenolic compounds epidemiologically proves to have an opposite relationship with the rate of morbidity and mortality caused by the coronary heart disease [13]. Consumption of polyphenolic-rich beverages in a short time increases the status of serum antioxidant in healthy subjects which is able to control cholesterol accumulation in macrophages [14].

One of the plants containing polyphenol compounds is *Mezzetia parviflora* Becc (*M. Parviflora*). In Buton island, located in Eastern Indonesia, the boiled wood bark of the plant is used to reduce cholesterol level. It is also used as a slimming medicine, antidiabetic, and antitumor. The effects are due to the ability of anti free-radical compound which is in a fairly high amount in the plant [15]. However, there is no scientific data that depict the antiatherogenic activity of *M. parviflora* and the underlying mechanisms. Therefore, the present work is designed in order to develop the *M. Parviflora* extract to become standardized herbal that has antiatherosclerosis effects.

EXPERIMENTAL SECTION

Preparing the Material and Extraction

The fresh stem bark of *M. parviflora* were collected locally from Buton island. After that, they were dried using oven in temperature of 40°C. The dried material was crushed up and ground to get homogeneous fine powder and then it was ready to be used as research material. A total of 5-kg of *M. parviflora* powder was extracted through maceration with ethanol-water solvent (7:3). The ethanolic extract was concentrated and evaporated until the extract was free from ethanol, and then lyophilized until dry extract was obtained (686.5 g). 500 g ethanolic extract of *M. parviflora* was subjected to a solid-liquid partition with acetone. The amount of acetone extract was 75 g and insoluble in acetone was 407 g. The acetone insoluble extract was standardized and used in the next test.

Animals and Experimental Design

The experimental protocols were approved by the Ethics Committee of the University of Hasanuddin, Indonesia (No. 0496/H04.8.4.5.31/PP36-KOMETIK/2010).

Activities of *M. parviflora* extract were tested by using male Wistar rat which were put in cages in a controlled environment. During the adaptation, rats were fed with pellet and tap water *ad-libitum* for 1 month until the rats weighing between 170-200g. The animals were randomly assigned to six experimental groups with three rats in each group. Animals in groups I served as positive control, was given simvastatin 3.6 mg/kg. Groups II, III, and IV were given sequentially 100, 300 and 500 mg/kg of *M. parviflora* extract peroral once a day. The agents were administered along with cholesterol 200 mg/kg administration. Group V, served as the control group, received cholesterol only. Rats in group VI, as the normal control, was not given any treatment. The test was performed for 3 months.

Measurement of Plasma Biochemistry

Before starting the treatment, animal plasma biochemical profiles were measured as a baseline. In the end of treatment, the same procedures were performed. After over night fasting (12 hours), rats were sacrificed under ether anesthesia and blood samples were collected intracardially into centrifuge tube. The serum were separated by allowing the coagulated blood to clot for 15 minutes at temperature of 25°C, centrifuged at 2500 rpm for 20 minutes, and then stored in plastic vials at -20°C until analysis of total cholesterol, triglycerida, HDL and LDL. Lipid peroxidation was estimated in serum by measuring the malondialdehyde (MDA) production generated in the thiobarbituric acid reaction. MCP-1 levels were only determined at the end of activities and measured by using rat Elisa kit (Invitrogen KRC1011).

Analysis of Antiatherosclerotic Effect

The thoracic aortae of these rats were rapidly and carefully dissected from *aorta ascendens arch tobifurkasi iliaka* and then washed with cold *phosphate buffer saline* (PBS) containing 1 mmol/liter EDTA. Atheroma observation was performed by immunohistochemistry staining method with anti-macrophage antibody (MAC387).

Immunohistochemistry

Consecutive 4 μm sections were cut from each block, deparaffinized with xylene and rehydrated with graded ethanol solutions in deionized distilled water. Serial sections were processed for the immunohistochemical study.

Immunohistochemistry was performed using the streptavidin-biotin-peroxidase method with labeled streptavidin-biotin (LSAB; Dako, Carpinteria, USA). Briefly, the sections were placed in a glass box filled with 10 mmol/L citrate buffer (pH 6.0) and were autoclaved for 15 min at 125°C to retrieve antigenicity. The sections were allowed to cool in the box at room temperature for 60 min before being immersed for 30 min in *peroxidase blocking reagent* (200 ml of methanol + 3.2 ml of H₂O₂ 30%) to block endogenous peroxidase, then immersed in *antigen retrieval solution* (*Target Retrieval Solution*, TRS, Dako) at 80°C and washed twice in PBS. The avidin-biotin blocking solution was applied to the sections for 30 min to block avidin in the tissue. The primary *monoclonal rat antibody, anti-macrophages*, macrophage marker (MAC 387)(1:100 dilution, St. Cruz Biotechnology) was applied to sections and incubated overnight at 4°C in a moist chamber. Subsequently, sections were incubated with biotinylated goat antirabbit IgG (Dako) for 30 min and streptavidin conjugated to horseradish peroxidase (Dako) for 30 min. Chromogenic fixation was carried out by immersing the sections in the solution of 3,3-diamino-benzidine tetrahydrochloride (DAB) (Dako) at room temperature for 10 minutes until a distinct reaction product was evident microscopically. The sections were then counterstained with Mayer's hematoxylin. Immunostaining of anti-MAC 387 antibody was graded according to the number of stained cells and the staining intensity in individual cells as follows: negative (score 0), almost no positive cells; low (score 1), 1-10% of the cell showed brown staining; and high (score 2), over 10% of the cells showed brown staining. Immunostaining was evaluated independently by three independent observers who were unaware of the clinical and histological diagnoses, and all of the sections were scored twice to confirm the reproducibility of the results.

Statistical Analysis

The data were statistically analyzed by using SPSS (Statistical Package for Social Sciences) software for windows version 15.0. The results were expressed as means \pm S.D. The statistical significance of the treatments analyzed with Kruskal-wallis test and the differences between the group means was determined using the Whitney Test. Differences are considered significant when $p < 0.05$ and very significant when $p < 0.01$.

RESULTS AND DISCUSSION

Antihyperlipidemic and anti-lipid peroxidation Activities

The most important risk factors of atherosclerosis and coronary heart disease (CHD) are advanced age, male gender, smoking, diabetes mellitus, hypertension, and hypercholesterolemia [16,17]. In this study, we investigated the antiatherogenic effect of *M. parviflora* extract in high cholesterol fed rats. First, the antihyperlipidemic and anti-lipid peroxidation effect of *M. parviflora* extract was obtained and the results can be seen in Table 1. These data showed that cholesterol intake (group V) for three months caused a significant increase in the total cholesterol (TC), triglycerides (TG), and *low density lipoprotein* (LDL) as well as the decrease of *high density lipoprotein* (HDL) when compared with normal control group (group VI).

The decrease of LDL level reduces cholesterol deposition in medium artery and aortae because the LDL has a role to carry cholesterol from the liver to periphery cells and artery muscle cells. The increase of HDL level helps to remove cells from periphery cells and to carry cholesterol back to the liver. Therefore, the increase of HDL level is expected, and so is the LDL level. Besides that, LDL in an oxidated form is caught by macrophage through a scavenger mechanism. Therefore, anti-arteriosclerotic medicines should be able to decrease VLDL and LDL and/or increase HDL.

The administrations of *M. parviflora* extract and simvastatin to the treatment groups were able to prevent a hyperlipidemia condition. They significantly inhibited the elevation of plasma lipid profiles induced by dietary cholesterol when compared with the cholesterol control group (group V) in dose dependent manner. There were no significant differences between *M. parviflora* extract and control drug (simvastatin) effects on LDL and HDL levels but not on total cholesterol and triglycerides levels. The ability of the extract to increase the HDL level and attenuate the LDL level causes the reduction of the atherogenic index (LDL/HDL) suggesting that the extract may help to prevent the progression of atherosclerosis [18, 19]. These results are in accordance with the results of El-Adawiet al.

[20] who reported that grape seed extract which was rich in polyphenol compounds showed an obvious hypocholesterolemic effect.

Table1. Test results of antihyperlipidemic and anti-lipid peroxidation effect of *M. parviflora* extract

GROUP	TREATMENT	Average Percentage of Total Cholesterol Decrease	Average Percentage of Triglycerida Decrease	Average Percentage of HDL-Cholesterol Increase	Average Percentage of LDL-Cholesterol Decrease	Average Percentage of MDA-Decrease
I	Cholesterol +Simvastatin	-40.31±4.46*	13.33±3.13*	126.7±15.7*	-25.12±1.57*	28.72±15.86*
II	Cholesterol+ Extract of <i>M. parviflora</i> 500mg/Kg	-21.71±3.33*	18.11±1.20*	144.3±58.1*	-19.88±5.47*	-43.41±15.73*
III	Cholesterol+ Extract of <i>M. parviflora</i> 300mg/Kg	-12.20±6.56*	24.81±1.27*	106.1±28.6*	4.53±3.39*	-27.09±9.25*
IV	Cholesterol+Extract of <i>M. parviflora</i> 100mg/Kg	-9.85±2.00*	43.56±12.16*	61.5±6.9*	11.87±0.65*	8.55±3.35*
V	Cholesterol (control)	43.09±3.11	76.56±5.97	-4.4±2.8	28.47±2.80	117.89±18.67
VI	Normal Control	3.75±2.01*	17.09±1.34*	40.0±13.9*	7.71±4.71*	15.33±7.64*

*Normal control group not given any treatment, control group received cholesterol 200 mg/kg only, the other groups were given the agents along with cholesterol 200 mg/kg administration. The test was performed for 3 months. *p<0.05 compared to control*

Hipercholesterolemia, one of the several main factors of atherosclerosis, increases the release of radicals in some ways. The main target of oxygen free radicals is to attack the double unsaturated fatty acid on membrane lipid to cause lipid peroxidation and consequently to produce the disorganization of cell structure and functions [9]. Efficiency of antioxidant protection in a hypercholesterolemic patient depends on the balance between oxygen free radicals and antioxidant availability.

Several studies have indicated that polyphenols (mainly catechins and procyanidins) of grape, wine, and grape seed extracts inhibit the oxidation of low-density lipoprotein (LDL). Tea antioxidants with a greater number of phenolic hydroxyl groups have greater antioxidant power, i.e., epigallocatechingallate (8 groups) >epicatechingallate (7 groups) > GC (6 groups) >epicatechin (5 groups) >epigallocatechin (6 groups)[21]. Polyphenol in tea also has *electron-donating* antioxidant characteristics whose activities are related with the amount and location of hydroxyl cluster that exists in the galloil cluster [22].

The *in vivo* study of lipid peroxidation inhibition of the extract was performed by measuring the malondialdehyd (MDA) levels as a final product of lipid peroxidation. Dietary cholesterol produced a significant increase in plasma MDA levels reaching up to 117.89% of the normal values. *M. parviflora* extract significantly inhibits the lipid peroxidation reaction in animal test dose dependently. In previous study we determined the *in vitro* LDL-oxidation inhibitory of *M. parviflora* extract. The result showed that 50 µg/ml *M. parviflora* extract prevented LDL oxidation induced by CuSO₄ by prolonging the LDL-oxidation lag time (unpublished data).

As a positive control, we used simvastatin. It has been reported that simvastatin has antiatherosclerotic character due to its antioxidant capacity [23]. In this experiment simvastatin showed a significant inhibitory effect on lipid peroxidation but its activity was lower than the *M. parviflora* extract. The high potency of *M. parviflora* extract as anti lipid-peroxidation, thereby, might be expected to responsible for a preventive effect on oxidative stress related cardiovascular diseases.

MCP-1 Levels

Arteriosclerosis is a response not only to hyperlipidemia but also to the inflammations of the vessel wall. The early stage of atherosclerosis is characterized by the focal attachment of monocytes to the endothelium and their subsequent transendothelial extravasation into the vessel wall [24, 25]. One of molecules that has chemotactic activity for monocytes is monocyte chemoattractant protein-1 (MCP-1) [26, 27].

The following is the graph of MCP-1 content measured in rat serum after being treated with cholesterol, cholesterol plus simvastatin and cholesterol plus *M. parviflora* extract with doses of 100 mg/Kg, 300 mg/Kg and 500 mg/Kg for three months.

Cholesterol feeding caused an increase in MCP-1 level that is higher compared to the normal animal group. It means that a high intake of cholesterol induced marked elevation of MCP-1 content. The MCP-1 levels of simvastatin and *M. parviflora* extract (500 mg/Kg) treated groups were lower than the normal group and the cholesterol control group. Sparrow *et al.* [28] investigated the anti-inflammatory and antiatherosclerosis effect of simvastatin. They

found that simvastatin administered orally to mice 1 hour before carrageenan injection reduced the extent of carrageenan-induced foot pad edema and simvastatin prevented an increase in aortic cholesterol content. A similar anti-inflammatory study conducted by Marketou, et al. [29] showed that simvastatin exerted inhibitory effects on oxidative stress, proinflammatory cytokines, and endothelial activation in hyperlipidemic subjects based on the results of serum concentrations of total peroxides (TP), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α) and soluble intercellular vascular adhesion molecule 1 (sICAM 1). In our study, MCP-1 was significantly decreased by *M. parviflora* extract 500 mg/kg-treated group, but not by *M. parviflora* extract at 100 mg/kg. This result indicated that *M. parviflora* extract effectively diminished MCP-1 release at a dose of 500 mg/kg, only. Our preliminary study showed that *M. parviflora* extract (100 ppm) significantly attenuated the in vitro secretion of monocyte chemoattractant protein (MCP)-1 and TNF-alpha [30].

Table2. The MCP-1 content Measured in Rat Blood Serum After Treatment for 3 months

GROUP	TREATMENT	Average of Total Serum MCP-1 (pg/mL)
I	Cholesterol +Simvastatin	64493.333 \pm 2759.879*
II	Cholesterol+ Extract of <i>M. parviflora</i> 500mg/Kg	68233.333 \pm 2557.368*
III	Cholesterol+ Extract of <i>M. parviflora</i> 300mg/Kg	71033.333 \pm 1107.309*
IV	Cholesterol+Extract of <i>M. parviflora</i> 100mg/Kg	71613.333 \pm 1248.092 ^{ns}
V	Cholesterol (control)	70293.333 \pm 1923.469
VI	Normal Control	72113.333 \pm 2955.763*

Normal control group not given any treatment, control group received cholesterol 200 mg/kg only, the other groups were given the agents along with cholesterol 200 mg/kg administration. The test was performed for 3 months. * $p < 0.05$ compared to control

Some researches suggest that polyphenols have anti-inflammatory effects through modulation of the expression of pro-inflammatory mediators such as cyclooxygenase, lipoxygenase, NO synthase, and several important cytokines (nuclear factor-kappa B and mitogen-activated protein kinase signaling) [31-34].

Antiatherosclerosis Activities

Endothelial dysfunction occurs before the structural manifestation of atherosclerosis and thus serves as an independent predictor of future cardiovascular events [7]. The endothelial activation can be recognized from early monocytes adhesion in the endothelial layer so the monocytes which is initially in the circulating monocyte system changing into resident monocyte and finally differentiate into macrophage. This condition is assessed through the immunohistochemistry staining. The marker used is macrophage marker (MAC 387), a monoclonal antibody, that detects Macrophage migration inhibitory factor (MIF)-Related Protein MRP8/MRP14. This marker has a cross reaction with activated leukocytes (neutrophils and monocytes) [35] and activated endothelial cells [36-38]. Therefore, the monocytes, leukocytes, and activated endothelial cells appear in this immunohistochemistry staining. The cross reaction is due to the presence of identical epitopes in the molecules, either in whole or in part [36]. This method is chosen because the previous study showing that 200 mg/kg BB cholesterol administration to rats every day for three months has not shown any formation of atherosclerotic plaque, so a marker is needed to be used for the early step of atherosclerosis occurrence.

The following is a microscopic description of immunohistochemistry coloring results:

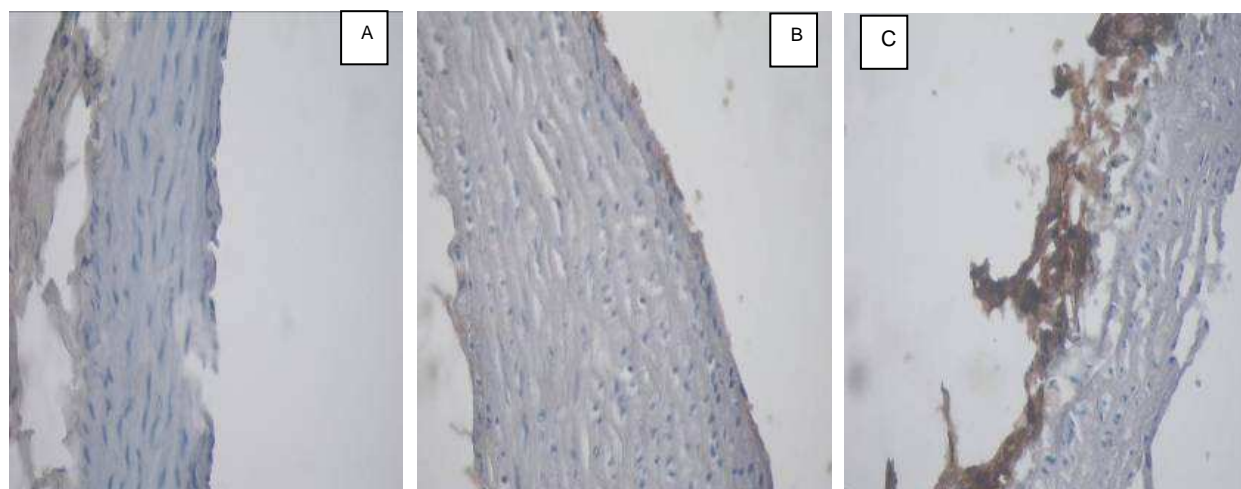


Figure 1. Endothelial Cell Immunohistochemistry Coloring

A. normal cells (Score 0) were shown by *M. parviflora* extract 500 mg/kg-treated group which was same with normal group and simvastatin (3.6 mg/kg)-treated group.
 B. 1% - 10% cell colored brown by monoclonal antibody used (Score + 1) was shown by *M. parviflora* extract 100 mg/kg-treated group.
 C. > 10% cell colored brown by monoclonal antibody used (Score + 2) was shown by the sick animal control group.
 Magnification 10 x 40

The results of immunohistochemistry staining show that a hypercholesterolemic diet caused an increase in the number of activated endothelial cell and in the amount of monocytes *resident* on the endothelial cell surface. It was scored as +2 and can be extrapolated as type I lesion in human atherosclerosis. The relationship between the score of endothelial dysfunction and the treatments was analyzed by using the contingency test and the chi-square value. The result showed that the value was larger (23.800) than the table value ($\chi^2_{0,05(12)} = 21.026$). It means that there was a relationship between endothelial dysfunction level and the treatments.

The administration of *M. parviflora* extract along with cholesterol can prevent the atherosclerosis occurrence in dose dependent manner. *M. parviflora* extract 500 mg/kg-treated rats did not show the presence of brown colored cells stained by monoclonal antibody the same as that in the healthy animal group. The same protecting effect was shown by the simvastatin (3.6 mg/kg) group. Meanwhile, the animal test group treated with lower dose of *M. parviflora* (100 mg/kg) showed that only 1-10% cells were stained by monoclonal antibody (score +1). No animals treated extract showed score +2 (>10% cells stained by monoclonal antibody) as in the sick animal control.

M. parviflora extract consists of condensed flavonoid and tannin [15]. Therefore, consumption of *M. parviflora* extract may cause accumulation of antioxidant active compounds in cells, preventing oxidation-induced cell damage and oxidized LDL-induced atherosclerosis plaque formation.

CONCLUSION

M. parviflora-standardized extract may resist the atherosclerosis process caused by the cholesterol intake through antihyperlipidemia and anti-lipid peroxidation activities.

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