Pharmacogn J. 2017; 9(2): 171-175
A multifaceted peer reviewed journal in the field of Pharmacognosy
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Original Article

Lipid Peroxidation Inhibitory Activity *In vitro* of *Mezzetia parviflora* Becc. Wood Bark Polar extract

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ABSTRACT

Introduction: The wood bark of *Mezzetia parviflora* Becc, has long served as one of the most important traditional herbal medicine sources in Buton Regency, Southeast Sulawesi. *M. parviflora* extracts were rich in polyphenols. This study was aimed to explore the lipid peroxidation inhibitory activity of polar extract of *M. parviflora*. **Methods:** The polar extract is the result of ethanol extract partition solved in acetone. The extract will keep polar components which are insoluble in acetone. Assayed methods applied are ß-carotene bleaching inhibition, thiobarbituric acid reactive substance (TBARS) measurement, and continuous monitoring of conjugated dienes formation in LDL. **Results:** M. parviflora extract inhibit ß-carotene/ linoleic acid oxidation, showed by IC50 value of 15.83 µg/ml in 30th minute; but the potency will be reduced to IC50 value of 111.19 µg/ml and 225.07 µg/ml after the 60th and 120th minute of incubation. *M. parviflora* extract inhibit MDA formation as for linoleic acid peroxidation product until the third day; at 20, 40, 60, 80 and 100 µg/ml inhibit MDA formation as many as 29.16 ± 2.41%, 4.24% ± 43.27, 54.08 ± 2.87%, 59.88 ± 1.90%, and 69.75 ± 2.32%, respectively. *M. parviflora* extract at 50 µg/ml can inhibit LDL-oxidation induced by CuSO4, performed by LDL-oxidation lag-time elongation until 70 minutes, similar ability was performed by epigallocathecin gallate at 5 µg/ml. **Conclusions:** *M. parviflora* extract expressed relatively strong protection against lipid and LDL oxidation which can serve as the scientific basis of its development as a remedy for various diseases caused by lipid peroxidation.

Key words: Conjugated diene, Low-density lipoprotein, Malondilaldehyde, *Mezzetia parviflora* Becc, Polyphenol.

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History
- Submission Date: 31-10-2016;
- Review completed: 08-11-2016;
- Accepted Date: 18-11-2016.

DOI : 10.5530/pj.2017.2.28

Article Available online
http://www.phcogj.com/v9/i2

INTRODUCTION

Free radicals, formed by some endogen metabolism reactions, can attract hydrogen atom from unsaturated lipid to begin membrane lipid peroxidation. The damage which begins by lipid peroxidation may cause cancer, cardiovascular disturb, and immunodeficiency. Our body produces molecules such as vitamin E and enzymes, such as SOD, catalase and peroxidase to control lipid peroxidation. Meanwhile the instability between endogen antioxidant and free radical production, may lead to "oxidative stress" and occurrence of chronic disease.[1-6] To prevent that situation, antioxidant is needed where generally come from plant rich of phenolic compound.[6-9]

Mezzetia is an indigenous plant in Andaman, Thailand peninsula, Malaysia peninsula, Sumatra, Kalimantan and Maluku.[10,11] *Mezzetia* consist of 4 species, one of them is *Mezzetia parviflora* which traditionally applied as a medicine in some diseases, such as tumor, asthma, hiper cholestrol, diabetes, and other diseases related to cell damages because of free radical activity. The previous research proof that *M. parviflova* scavenge DPPH free radical effectively where IC_{50} which is insoluble in acetone extract, ethanol extract and acetone extract respectively 21.79, 30.22, 60.73, and 262.55 µg/ml. Insoluble in acetone extract performed higher antioxidant activity level than ascorbic acid (IC_{50}=30.22 ug/ml). High percentage of antioxidant activity related to phenolic condensed tannin in acetone extract that is 26.46 ± 0.315 mg/mg of the extract (calculated as quebracho tannin).[12]

This research conducted to evaluate lipid anti-peroxidation activity of *Mezzetia parviflora* Becc. Wood bark polar extract. The applied assayed methods are β-carotene bleaching inhibition, thiobarbituric acid reactive substance (TBARS) measurement, and continuous monitoring of conjugated dienes (malondialdehyde-MDA) formation in LDL. The result may become a scientific proof about *M. parviflora* extract usage in solving lipid oxidation disease such as atheroschlerosis.

MATERIALS AND METHODS

The UV-Visible spectrophotometer (*Hewlett Packard*) was used to measure the absorption value. β-Carotene, LDL, linoleic acid, and 1,1,3,3-tetramethoxypropane (MDA) were purchased from Sigma Aldrich; α-tocopherol, thiobarbituric acid, trichloroacetic acid, $CuSO_4$, HCl, ethanol, and acetone were an analytical grade from E-merck.

Wood bark Collection and Extraction

Mezzetia parviflora Becc. wood bark were collected from Buton regency, Southeastern Sulawesi province and identified at Herbarium Bogoriense, Bogor. *M. parviflora* wood bark powder was extracted by eth-

Cite this Article: Murdifin M,Pakki E, Alam G, Manggau MA, Muslimin L, Rusdi M, Wahyudin E. Lipid Peroxidation Inhibitory Activity *In vitro* of *Mezzetia parviflora* Becc. Wood Bark Polar extract. Pharmacogn J. 2017;9(2):171-5.

anol 70% with maceration method and the solvent was evaporated by rotary evaporator then freeze dried. Ethanol extract was partitioned by acetone, partition were finished when acetone gave no color and KLT perform visible appearance difference between soluble and insoluble acetone extract.

Lipid Peroxidation Inhibitory Test

Inhibition of lipid peroxidation based on the extract's ability to protect linoleic acid from heat-induced oxidation was assessed. Two methods were used to describe the effect of *M. parviflora* extract: the inhibition of β-carotene bleaching method and inhibition of thiobarbituric acid reactive substance (TBARS) formation.

β-carotene bleaching inhibition method

This assay conducted based on the measurement of β-carotene oxidative bleaching in β-caroten/linoleic acid mixture with and without the addition of *M. parviflora*, the method described by Kikuchi and Kitamura (1987) with a slight modification.[13] Briefly, 6.0 mg ß-carotene was dissolved in 10 ml of chloroform, then 1 ml of solution pipetted to glass filled of 20 mg linoleic acid. 5 ml of mixture then pipetted to reaction tube filled of extract in various concentration, mixed homogenously. Sample absorptions were conducted before and after incubation at 50°C for 30, 60, and 120 minutes. β-carotene bleaching inhibition percentage was calculated by the following formula:

% inhibition = $[1- (AA_{(120)} - AC_{(120)}) / (AC_{(0)} - AC_{(120)})] \times 100$

$AA_{(120)}$: sample absorbance at t = 30, 60 or 120 minute

$AC_{(120)}$: control absorbance at t = 30, 60 or 120 minute

$AC_{(0)}$: control absorbance at t = 0 min

The percentage of inhibition of β-carotene bleaching produced by each concentration of extract then tabulated. The IC_{50} (Inhibitory Concentration 50%) value was calculated by using probit analysis.

Thiobarbituric Acid Reactive Substance (TBARS) Formation Inhibition Method

Malondilaldehyde (MDA), the last product of linoleic acid oxidation, was measured with thiobarbituric acid reactive substance (TBARS) method. Briefly, 20-100 µg/ml of *M. parviflora* extract dilution were prepared with water as a solvent. 4000 µl of this solution was mixed by 1000 µl of linoleic acid (13 g in 100 ml) incubated in shaking water bath. Extract mixture combine with 2.5 ml thiobarbituric acid (TBA) solution which contain 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. The mixture then was boiled for 10 minutes until its color was changed into pink. The mixture then centrifuged at 5000 g at 25°C for 10 min. Supernatant absorbance measured at 532 nm. Standard curve obtained 1,1,3,3-tetramethoxypropane (MDA) from 0 to 10 ppm concentration and TBARS stated as mg MDA/kg sample.[14-16] Measurement continued after incubating for one to five days. Lipid peroxidation inhibition percentage was calculated by the following formula:

% inhibition = $100 [(A_{Control} - A_{sample}) / A_{control}]$

A_{sample} : sample absorbance

$A_{control}$: control absorbance

$CuSO_4$-induced LDL-oxidation *in-vitro* inhibition

Inhibition of LDL oxidation was measured based on the prolongation of lag time of conjugated diene formation. Extract were divided into series then incubated with LDL (Sigma Aldrich) for 18 hours at 37°C in water bath, in plastic tube of diameter 1 cm by incubating the extract, LDL (100 mg protein/L) and $CuSO_4$ (5 µmol/L). Diena conjugated formation monitored continously by measuring its absorption at 234 nm,[17-19] each 10 min interval time for 360 minutes by UV-VIS spectrophotometer.

RESULTS AND DISCUSSION

β-carotene bleaching inhibition method

β-carotene bleaching inhibition method was measured based on the ability of an antioxidant to inhibit orange color reduction of β-carotene due to the oxidation occurred in linoleic acid/β-carotene mixture.[13,20-22] β-carotene is very sensitive to free radical formed by linoleic acid oxidation. Linoleic acid free radical formed when boiled will attract hydrogen atom of methylene diallylic, then formed peroxide fee radical forces conjugated double bond of β-carotene which is responsible for its carotenoid orange color which span at 400-500 nm.

The result indicates that *M. parviflora* extract inhibited β-carotene/linoleic acid oxidation and progressive activity occurring along with extract concentration. But, this activity was weaker than Vitamin E activity. IC_{50} value (15.83 µg/ml) indicated that the extract inhibited linoleic acid oxidation in 30 minutes. The value was two times weaker than vitamin E (6.77 µg/ml). The extract potency at the 60th - 120th minute was sharply decrease i.e. 19 times (111.19 µg/ml) and 25 times (225.07 µg/ml) as weak as vitamin E (Table 1). The difference of extract polarity and β-carotene is the reason of that phenomenon. Recent studies reported that lower polarity of vitamin E result in better dissolution in lipid phase and more efficient in protecting linoleic acid.[23,24]

Table 1: Inhibition of β-carotene bleaching by *M. parviflora* extract compared to Vitamin E

Sample	Inhibition of beta-carotene bleaching (%)		
	30 min	60 min	120 min
M. parviflora extract			
100 µg/ml	65.10 ± 1.02	47.29 ± 1.65	35.01 ± 6.27
80 µg/ml	61.59 ± 5.49	38.29 ± 2.36	34.46 ± 3.13
60 µg/ml	59.49 ± 5.27	31.27 ± 3.93	29.91 ± 4.31
40 µg/ml	57.77 ± 4.30	18.94 ± 3.42	22.33 ± 4.58
Vitamin E			
10 µg/ml	86.01 ± 3.29	74.79 ± 1.20	59.72 ± 3.17
8 µg/ml	70.112.00 ±	64.21 ± 4.22	42.36 ± 4.87
6 µg/ml	36.77 ± 4.66	48.86 ± 3.95	19.49 ± 9.15
4 µg/ml	29.32 ± 5.50	35.05 ± 2.88	5.09 ± 0.27

Anti-Lipid-Peroxidation with Thiobarbituric Acid Reactive Substance (TBARS) Method

The principle of this method is the reaction of one molecule of malondil aldehyde (MDA) and two molecules of TBA to form malonaldehyde-TBA complex. There will be change into red in color and the fluorescence will be absorbed at about 500 nm using visible spectrophotometer.[24]

The graph below showed the ability of *M. parviflora* extract inhibiting linoleic acid peroxidation reaction within a period of 5 days compared to the blank.

Percentage of lipid peroxidation of linoleic acid at the third day performed that *M. parviflora* extract at the concentration of 20, 40, 60, 80 and 100 µg/ml indicated inhibition 29.16 ± 2.41%, 43.27 ± 4.24%, 54.08 ± 2.87%, 59.88 ± 1.90%, and 69.75 ± 2.32% respectively. The antioxidant activity, however, declines rapidly on the fourth day, indicated by a sharp increase in absorbance MDA after 3 days (Figure 1).

Antioxidant compound in the extract bond to free radicals formed in initial reaction, furthermore it can inhibit continuous reaction between free radical oxygen which produce reactive radical peroxide. Antioxidant neutralize radical peroxide by releasing hydrogen atom then stabilizing the radical substance during oxidation.[25]

$CuSO_4$-induced LDL-oxidation inhibition

Not only linoleic acid peroxidation method but also lipid anti-peroxidation assay was conducted directly to cholestrol-LDL, because LDL oxidizes together with another oxidized lipid contribute to ateroschlerosis patophisiology through various mechanisms; include proinflamatory, imunogenic and cytotoxic.[26,27]

Activity assay of LDL-oxidation inhibition in this study conducted by observing the LDL-oxidation kinetic that is monitoring conjugated diena formation continously after LDL-oxidation induction by $CuSO_4$. LDL-oxidation kinetic pictures three phases of oxidation, namely, lag phase, propagation phase and decomposition phase. Lag phase is an initial phase that is interval phase between $CuSO_4$ addition and a quick LDL-lipid oxidation process. Initial addition of $CuSO_4$ lipid oxidation reaction occurs slowly because antioxidant activity will protect lipid from free radical attack. Meanwhile, at the end of lag phase, the antioxidant decrease the lipid peroxidation product increase which indicated by significant increasing absorbance of conjugated diena at propagation phase. However, at decomposition phase, absorbance decrease slowly because diena conduct continuous lipid peroxidation reaction. Lag phase will be reached by determining intercept of lag phase and propagation phase.[28]

This is the graphic related to time (minute) and absorbance of conjugated diena at 234 nm after LDL-oxidation induced by $CuSO_4$:

Figure 2 indicates that oxidation in non-addition of antioxidant (blank) occur faster, can be seen from lag time occur at the 20th minute. Addition of *M. parviflora* extract 50 µg/ml and epigallocatekhin gallat (EGCG) 5 µg/ml perform the ability to inhibit oxidation velocity showed by 70 min lag time. Even though *M. parviflora* extract and EGCG indicate similar LDL-oxidation lag time but absorbance of EGCG is higher than *M. parviflora* extract. It means that conjugated diena formed at the treatments and EGCG are higher than extract formed.

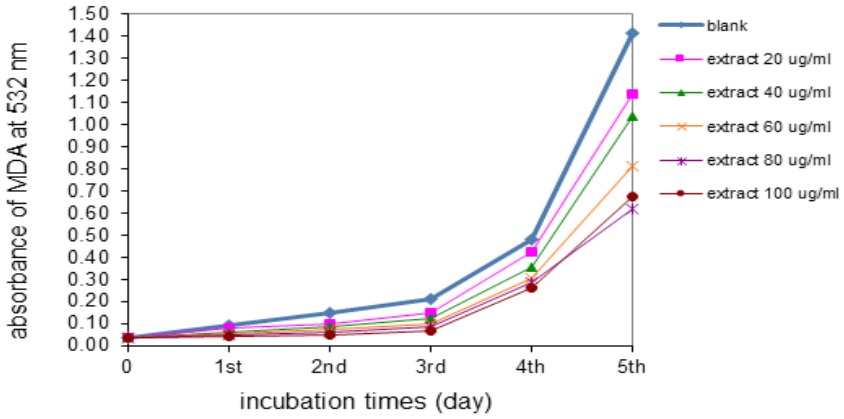

Figure 1: *M. parviflora* Extract Activity against linoleic acid peroxidation reactions During Interval Time 5 Days Using TBARS Method. The end product of linoleic acid oxidation was measured as MDA.

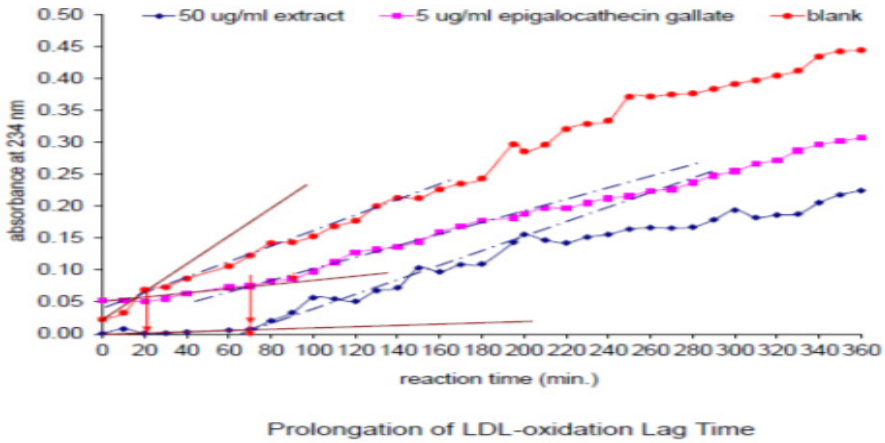

Figure 2: LDL-oxidation profile measured as conjugated diena with or without the addition of *M. parviflora* extract and epigalocathecin gallate.

Normally, LDL receptor recognize specific domain with positive charge among lysine residues, arginine and histidin at apo B. But, aldehyde interaction with amino group at apoB-100 make LDL have more negative charge then its affinity to LDL receptor decrease conversely will increase the affinity of scavenger receptor[3] that LDL susceptible to be caught by macrophage produce foam cell.[29] Foam cell accumulation develops into atherosclerosis lesion. Oxidized-LDL together with other oxidize lipid products contribute to atherosclerosis pathophysiology through certain mechanisms include pro-inflammatory characteristic, immunogenic or cytotoxic.[30]

Polyphenol prevent atherosclerosis through its mechanism in inhibiting LDL-oxidation, blocking lipid peroxidation and catching oxygen free radicals. The study of *Sanches-Moreno et al*. (2000) indicates that polyphenols are condensed tannins (tannin acid), flavonols (catechin, quercetin, rutin), cinnamic acids (caffeic and ferulic acid), stilbenes (resveratrol), benzoic acids (gallic acid), anthocyanidins (malvidin) are more able to inhibit oxidize LDL than Vitamin C and vitamin E.[17] Parameter applied by Sanches-Moreno is CLT_{50} that is a concentration which cause Lag time elongation of oxidize LDL as many 50% as the blank. Benzie dan Szeto (1999) stated that cathecin in tea inhibit LDL oxidation *in-vitro* and *in-vivo* based on the respective potency as follows: epigalocatechin (EGC) <epicatechin (EC) <epicatechin galat (ECG) <epigalotechin error (EGCG).[31] Another group compounds such as sterol ergostarol isolated from Pleurotus ostreatus fungi can inhibit lipid peroxidation.[32] The ability to inhibit lipid peroxidation associated with anticancer activity,[1,6] atherosclerosis,[3-5] antiinflammation,[33] and immunostimulant.[2] Furthermore, the ability of *M. parviflora* extract to inhibit lipid peroxidation is an important mechanism which contributes to defense ability against various diseases caused by this process.

CONCLUSIONS

Based on the research, concluded that *M. parviflora* extract at 20 to 100 µg/ml can inhibit lipid peroxidation reactions, both on the β-carotene bleaching test and the TBARS method. The extract 50 µg/ml can inhibit LDL-oxidation showing the lag-time lengthening of LDL-oxidation from 20 min to 70 min, the same activity was shown by epigallocatechin gallate 5 ug/ml.

ACKNOWLEDGEMENT

The study was supported by The Department of National Education of Republic of Indonesia for the Research Grants.

CONFLICT OF INTEREST

All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

ABBREVIATION USED

IC_{50}: inhibition concentration 50%; LDL: low density lipoprotein; MDA: malondilaldehyde; TBARS: thiobarbituric acid reactive substance; UV-VIS: ultra violet – visible.

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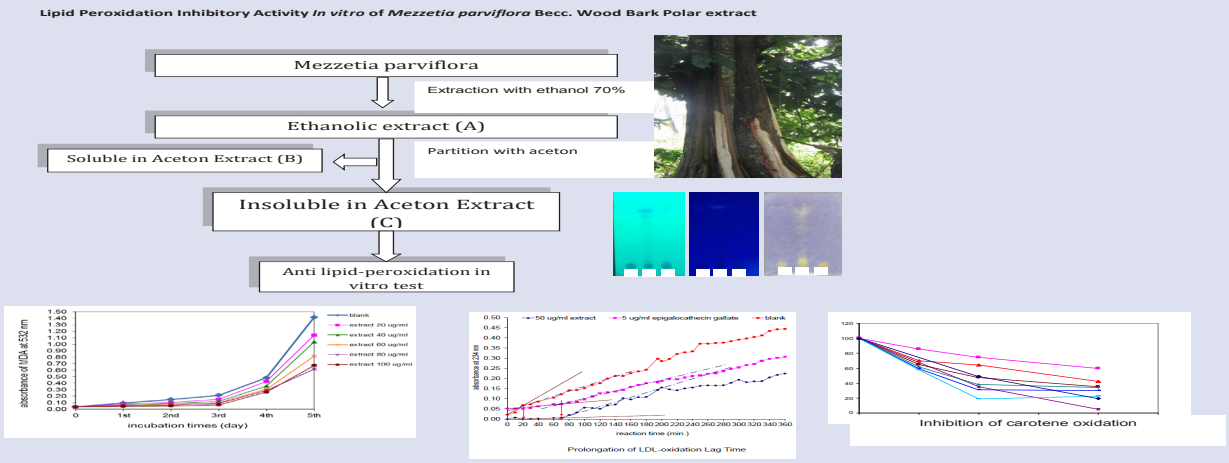
PICTORIAL ABSTRACT



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Prof. Dr. Elly Wahyudin : Has obtained his Ph.D degree in 1991 from Universite De Montpellier I, France. She is head of Biofarmaka Laboratorium, Pharmacy Faculty, Hasanuddin University Indonesia. Its main research fields are pharmacology, especially the study of the pharmacokinetics of a drug substance.Prof Elly was also much involved in research pharmacological activity of medicinal plants in eastern Indonesia.

Cite this Article: Murdifin M,Pakki E, Alam G, Manggau MA, Muslimin L, Rusdi M, Wahyudin E. Lipid Peroxidation Inhibitory Activity *In vitro* of *Mezzetia parviflora* Becc. Wood Bark Polar extract. Pharmacogn J. 2017;9(2):171-5.