INHIBITION OF HUMAN LOW-DENSITY LIPOPROTEINS OXIDATION BY *Hibiscus radiatus* CUV. CALYCES EXTRACT

Hernawan *

Technical Implementation Unit for Development Chemical Engineering Processes, Indonesian Institute Of Sciences
Yogya-Wonosari Street 32nd Km, Gading, Playen, Gunungkidul, Yogyakarta, 55861

Received 23 October 2007; Accepted *19 January 2007*

ABSTRACT

Hibiscus radiatus Cuv *calyces extracts rich in polyphenols was screened for their potential to inhibit oxidation of human low-density lipoproteins-cholesterol (LDL-C)* in vitro*. The inhibition of LDL-C oxidation (antioxidant activity) was determined by measuring the formation of conjugated dienes and thiobarbituric acid reagent substances (TBARS). LDL-C oxidation was carried out in the presence of* H. radiatus *Cuv calyces extract (20 and 50 µM). $CuSO_4$ (10 µM) was used as the oxidation initiator and butylated hydroxytoluene (BHT) at 50 µM was used as standard antioxidant. The protective effect of* H. radiatus *Cuv. calyces extract toward human low-density lipoproteins, complex lipid system was demonstrated by significant increase lag time (≥ 103 min), diminished of the propagation rate (44 %), and diminution of conjugated dienes formation 59.42 % (50 µM) compared to control.*

Keywords: *antioxidant, conjugated dienes,* Hibiscus radiatus *Cuv, low-density lipoproteins-cholesterol*

INTRODUCTION

The search for potential natural antioxidants, especially from plants sources, as nutritional supplement, health food, and phytomedicine has become an important research issue at a world-wide level. Many researchers and physician now contemplate the use of antioxidant treatments as a key strategy for inhibiting or reversing the process of carcinogenesis [1]

Antioxidant components are microconstituents present in the diet that can delay or inhibit lipid oxidation, by inhibiting the initiation or propagation of oxidizing chain reactions, and are also involved in scavenging free radicals. Reactive Oxygen Species (ROS), e.g., superoxide radicals, hydroxyl radicals, and hydrogen peroxide, have been proposed as significant causative factors in some radical mediated conditions including aging [2], cancer [3], and cardiovascular disease [4].

H. radiatus Cuv. (*Malvaceae* family) is a tropical plant. It is cultivated in warm countries. Its flowers are yellow with red calyces. The calyces are commonly used as a substrate for herbal teas and refreshing drinks. Previous studies had demonstrated that *H. radiatus Cuv.* calyces extract showed an antioxidant activity toward linoleic acid peroxidation [5]. Thus, this study was carried out to evaluate the antioxidant capacity of *H. radiatus Cuv.* calyces extract toward human low-density lipoprotein-cholesterol oxidation.

EXPERIMENTAL SECTION

Chemical Reagents

All of reagents are analytical grade. Tween 20 (polyoxyethylenesorbitan monolaurate), $CuSO_4$, BHT (Buthylated hydroxytoluene), sodium phosphate buffer pH 7.0, TBA (Thiobarbituric Acid), Na_2CO_3, ethanol, methanol, ethylenediamintetra-acetic (EDTA), and Folin-Ciocalteau reagent.

Plant Material

The object of the study were the calyces of *H. radiatus Cuv.* collected from Gading, Playen, Gunungkidul, Yogyakarta. Taxonomic identification was performed by Dr. Eko Baroto Waluyo (Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences)

Procedure

Preparation of calyces extract

Fresh *H. radiatus Cuv.* calyces were washed and dried. The dried samples were cut into small pieces and soaked in ethanolic aqueous solution at room temperature for 10 days. The extract was decanted, filtered under vacuum, concentrated in rotary evaporator under reduced pressure at room temperature. The crude concentrated extract stored at 20 ^{o}C until use

Determinations of total polyphenol compounds in calyces extract

The amount of total polyphenolic compounds was determined by oxidation-reduction colorimetric method described by Taga [6] using Folin-Ciocalteau reagent. Briefly, samples and standards were prepared in acidified (0.3% HCl) methanol-water solution (60:40). One hundred microliter of this solution was added to 2 mL of 0.2% $NaCO_3$. After 2 min., 100 µL of Folin-Ciocalteau reagent/methanol (v/v) reagent was added to start reaction at room temperature (25 ^{o}C). Absorbance (λ_{max} = 750 nm) was measured after 30

* Tel/Fax : +62 274 392570; Email address : hern001@lipi.go.id

min. using a UV/Vis spectrophotometer. The phenolic concentrations were expressed as phenol equivalent by comparing with standard calibration curve using phenol solution (0.01-1 mg/mL)

Isolation of Low-Density Lipoproteins

Human low-density lipoproteins (1,019 < LDL < 1,063) were isolated from freshly prepared heparinized plasma obtained from healthy male donors (28 years). according to the method of Sattler [7] using ultracentrifuge (40,000 rpm, 12 h, at 15 °C). After separation LDL was dialyzed overnight at 4 °C with 0.01 M sodium phosphate buffer (pH 7). For oxidation experiments, the LDL dialyzed solutions were adjusted by dilution to 100 µg/mL

Measurement of conjugated dienes

The conjugated dienes (CD) was determined by measuring the absorbance at 234 nm according to the modified technique described by Esterbauer [8]. Briefly, 7.5 µM linolec acid emulsified with tween 20 (0.1%, v/v) mixture, in 10 mM phosphate buffer pH 7.0, control was incubated alone, or with *H. radiatus Cuv.* extract (20 and 50 µM). Oxidation was initiated by addition of 10 µM freshly prepared $CuSO_4$, and stoped by cooling in ice bath in the presence of 100 µM EDTA ang 20 µM BHT. The absorbance reading were taken every 15 min. over 240 min. at 37 °C in UV-Vis Spectrophotometer

$$\% \text{ inhibition} = \frac{A_c - A_s}{A_c} x100$$

Where A_c is absorbance of the control reaction, and A_s is absorbance of the treated sample. The peroxidation kinetic parameters: lag time (min), maximal rate of oxidation (nM/min), and maximal amount of CD formation (µM) were calculated using molar extinction coeficient of 29500 $M^{-1}cm^{-1}$

RESULT AND DISCUSSION

Fresh *H. radiatus Cuv.* calyces were washed and dried at 40 °C for 12 hours to remove the contaminant. The dried samples were cut into small pieces and soaked in ethanolic aqueous solution at room temperature for 10 days. After 10 days, the extract was decanted, filtered under vacuum, concentrated in rotary evaporator under reduced pressure. It yielded 9.7 % dark red concentrated extract.

Determination of total polyphenolic compounds in the *H. radiatus Cuv.* calyces extract using Folin-Ciocalteau reagent showed that the extract contains high amount of total polyphenolic compounds (1202.3 mg of phenol equivalent per 100 g extract). This result led us to suggest that these substances could responsible of the antioxidant properties of the extract. Polyphenol were reported to have an important role to stabilize lipid peroxidation [9] and are associated with a wide range of biological activity including antioxidant properties [10] due to their redox properties, as reducing agent, or hydrogen atom donors.

In order to confirm the protective action of *H. radiatus Cuv,* calyces extract on linoleic acid oxidation as reported in the previous studies [5], we tested its effect on human low-density lipoproteins-cholesterol oxidation by quantifying CD formation. Inhibition lipid peroxidation of human low-density lipoproteins (LDL-C) or antioxidant activity of *H. radiatus Cuv.* calyces extract was determined in the oxidation reaction of human low-density lipoproteins- oxidation initiated by Cu_2SO_4 monitored by conjugated dienes formation. Conjugated dienes formation was assessed using thiobarbituric acid reagent substances (TBARS). BHT was used as an antioxidant standard.

The obtained results showed that *H. radiatus Cuv.* calyces extract exhibit a significant inhibition of human low-density lipoproteins-cholesterol oxidation as assessed by conjugated dienes formation, as shown in Fig 1. The extends of inhibition of CD formation were 10,14 % and 59,42 % respectively at 20 µM and 50 µM, while BHT used as standard antioxidant at 50 µM, gave 60,32 %.

The effect on kinetic parameters of oxidation (Table 1), showed that *H. radiatus Cuv.* calyces extract prolonged the lag time (≥ 103 min.), diminished the propagation rate (44 %), and inhibited the maximal amount of CD formation 59.42 % (50 µM). The antioxidant effect of *H. radiatus Cuv.* calyces extract have seems in protecting human LDL-C (complex lipid system) and linoleic acid (simple lipid system), as described in the previous works [5] but more effective on inhibit linoleic acid oxidation than human low-density lipoproteins-cholesterol oxidation.

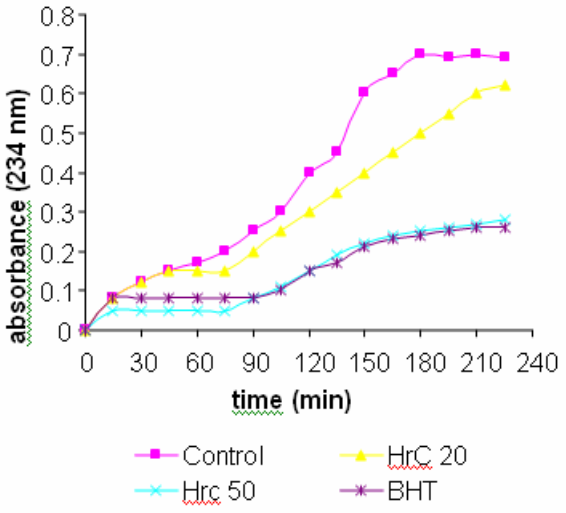

Fig 1. Effect of *H. radiatus Cuv.* calyces extract on Cu^{2+} induced human low-density lipoproteins oxidation monitored by conjugated dienes (CD) formation. Each point represents the mean of three replicates.

Table 1. Effect of *H. radiatus Cuv.* calyces extract on Cu^{2+} induced human low-density lipoproteins oxidation monitored by conjugated dienes (CD) formation.

Kinetic parameters	Treatments			
	Control	H r C 20	H r C 50	B H T
Lag phase(min)	78 ± 1,63	89 ± 0,82	103 ± 2,45	106 ± 1,63
Propagation rate (nM/min)	89 ± 0,82	70 ± 2,45	50 ± 0.82	48 ± 1,63
$[CD]_{max}$(mM)	20,35 ± 0,29	18,29 ± 0,24	8,26 ± 0,22	7,67 ± 0,55
% inhibition $[CD]_{max}$	0	10,14 ± 0,11	59,42 ± 0,34	62,32 ± 0,26

Note : Each value represents the mean ± SD of three replicates

CONCLUSION

The protective effect of *H. radiatus Cuv.* calyces extract toward complex lipid system, human low-density lipoproteins-cholesterol (LDL-C) was demonstrated by significant increase lag time ($\geq$ 103 min), diminished of the propagation rate (44 %), and diminution of conjugated dienes formation 59. 42 % (50 µM).

ACKNOWLEDGEMENT

The authors thank to Dr. Putut Irwan Pudjiono, M. Sc. the useful comments and Dr Eko Baroto Waluyo (Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences) for taxonomic identification.

REFFERENCES

1. Shireqi, I., Reddy, P., Brenner, D.E., 2000, *Crit. Rev. Oncol/Hematol*, 33, 157-167
2. Finkel, T., and Holbrook, N.J., 2000, *Nature,* 408, 239-247
3. Pietta P.G., 2000, *J. Nat. Prod.,* 63, 1035-1042
4. Frei, B., 1995, *Crit. Rev. Food. Sci. Nitr*:, 35, 83-98
5. Hernawan, and Angwar M., 2007, Study on In Vitro Antioxidant Effect of *Hibiscus radiatus Cuv*. Calyces Extract. *Proceeding of National Seminar on Sciences and Technology 2007*, Lampung University
6. Taga, M.S., Miller E.E., Pratt, D.E., 1984, *J. Am. Oil Chem. Soc*., 61, 928-931
7. Sattler, W., Mohr, D., Stocker, R.,1994, *Methods in Enzymol*:, 233, 469-489
8. Esterbauer, H., Streigl, G., Puhl, H., Rothenender, M., 1989, *Free. Radic. Ras. Commun.*, 6, 67-75
9. Duh, P.D., Tu, Y.Y., Yen, G.C., 1999, *Lebenmittel-Wissenschaft und Technogie*., 32, 269-277
10. Roginsky, V., 2003, *Arch Biochem Biophys.*, 414, 261-270