Antioxidant Effects of Aqueous Extracts from Dried Calyx of *Hibiscus sabdariffa* Linn. (Roselle) *in Vitro* Using Rat Low-Density Lipoprotein (LDL)

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The present study quantitatively investigated the antioxidant effects of the aqueous extracts from dried calyx of *Hibiscus sabdariffa* Linn. (roselle) *in vitro* using rat low-density lipoprotein (LDL). Formations of the conjugated dienes and thiobarbituric acid reactive substances (TBARs) were monitored as markers of the early and later stages of the oxidation of LDL, respectively. Thus, we demonstrated that the dried calyx extracts of roselle exhibits strong antioxidant activity in Cu^{2+}-mediated oxidation of LDL ($p<0.05$) *in vitro*. The inhibitory effect of the extracts on LDL oxidation was dose-dependent at concentrations ranging from 0.1 to 5 mg/ml. Moreover, 5 mg/ml of roselle inhibited TBARs-formation with greater potency than 100 μM of vitamin E. In conclusion, this study provides a quantitative insight into the potent antioxidant effect of roselle *in vitro*.

Key words *Hibiscus sabdariffa*; roselle; low-density lipoprotein (LDL) oxidation; antioxidant

Hibiscus sabdariffa Linn. (roselle) is widely cultivated in tropical areas and its red persistent calyx is the major component possessing a sour taste that is used as beverage and food colorants. It contains many chemical constituents including alkaloids, L-ascorbic acid, anisaldehyde, anthocyanin, β-carotene, β-sitosterol, citric acid, cyanidin-3-rutinoside, delphinidin, galactose, gossypetin, hibiscetin, mucopolysaccharide, pectin, protocatechuic acid, polysaccharide, quercetin, stearic acid and wax. As a traditional medicine, it is claimed to be effective against kidney stones and urinary bladder stones.[1,2] It is also used for its antibacterial, antifungal, hypocholesterolemic, antispasmodic and antihypertensive actions.[3—6]

Numerous studies have linked the elevation of plasma low-density lipoprotein (LDL)-cholesterol (LDL-C) level with the increased incidence of atherosclerotic events. LDL particles undergo extensive lipid peroxidation, resulting in generation of oxidized LDL and formation of atherosclerotic lesions.[7—11] Predictably, antioxidants such as α-tocopherol and probucol have been found to reduce LDL oxidation.[12—15] Various antioxidant constituents have been found in the calyx of *Hibiscus sabdariffa* Linn., including hibiscus anthocyanin, quercetin, L-ascorbic acid and protocatechuic acid (PCA). Antioxidant effects of roselle extracts have been investigated in many experimental models,[16—19] however the antioxidant effects of *Hibiscus sabdariffa* Linn. on LDL oxidation have so far not been fully determined. The present study was designed to quantitatively investigate the antioxidant effect of roselle on the oxidative modification of LDL induced by $CuSO_4$ *in vitro* by monitoring the formation of conjugated dienes and the formation of thiobarbituric acid reactive substances (TBARs).

MATERIALS AND MATHODS

Plant Material and Extracts The dried calyxes of roselle were blended to a fine powder and one kilogram of powders was extracted with 1 l of water and filtered through filter paper by suction. The filtrates were pooled and dried at 50 °C in a rotary evaporator. The average yield of the extract was 45.0%. The extracts were stored in a dark, moisture-free container at 4 °C. The antioxidant effect of PCA, a polyphenolic compound, has been investigated and found to exhibit potent action in primary cultures of rat hepatocytes induced by *tert*-butylhydroperoxide (*t*-BHP).[18] Therefore, in the present experiment, PCA content in the calyx of roselle was used for the standardization of batches; 25 mg of dried calyx extracts contained 10.98 μg of PCA. For this experiment, 150 mg of the crude extracts were dissolved in 1 ml of distilled water.

Reagents Disodium ethylene diamine was purchased from Mallinckrodt Inc. (St. Louis, MO, U.S.A.) and sodium chloride, sodium carbonate and sodium hydroxide from Merck (Haar, Germany). Diethylether was purchased from BDH laboratory supplies (Poole, England). Thiobarbituric acid, potassium bromide, disodium phosphate, sodium phosphate, sodium dodecyl sulfate, copper sulfate, folin & ciocalteu's phenol reagent, bovine serum albumin, α-tocopherol (vitamin E), trichloroacetic acid and butylated hydroxytoluene were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Isolation of LDL Blood was obtained from male Sprague-Dawley rats (National Laboratory Animal Center, Mahidol University, Thailand), weighing between 250—300 g. Rats were fasted overnight and blood was obtained by cardiac puncture under light anesthesia with diethylether.

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Blood was collected in glass tube with disodium ethylenediamine (Na_2EDTA) (final concentration of 1 mg/ml). Plasma was freshly separated by centrifuging blood samples at 3600 rpm at 4 °C for 10 min. LDL, density of 1.019—1.063 g/ml, was isolated from freshly prepared plasma by sequential ultracentrifugation technique. 0.5 ml of plasma was adjusted to a density of 1.006 by addition of 0.5 ml of the KBr solutions and centrifuged at 80000 rpm at 16 °C for 4.5 h using a Beckman TLA 100-2 rotor in a Beckman TL-100 ultracentrifuge. The VLDL fraction was recovered and the remaining solution was adjusted to a density of 1.063 with KBr solution. The LDL fraction was further purified by centrifugation at 80000 rpm for 5.5 h at 16 °C. The extracted LDL was dialyzed against 10 mM phosphate buffer saline (PBS) pH 7.4 at 4 °C for 24 h.[20] Protein concentrations in LDL samples were determined by the modified method of Lowry[21] using bovine serum albumin as a standard.

Continuous Monitoring of Formation of Conjugated Dienes in LDL LDL was adjusted to 100 μg/ml of LDL protein with 10 mM PBS pH 7.4 and incubated at 30 °C for 1 h with various concentrations of dried calyx extract of *Hibiscus sabdariffa* Linn. (0.001, 0.01, 0.1, 0.25 μg/ml). Vitamin E was dissolved in ethanol, dried under nitrogen and incubated with LDL solution (final vitamin E concentration of 100 μM). Oxidative modification of LDL was initiated by addition of freshly prepared 10 μM $CuSO_4$ solution at 30 °C in water bath for 6 h. Conjugated dienes formation during oxidation of LDL was continuously monitored by the spectrophotometric method of Esterbauer *et al*.[22] based on changes in absorbance at 234 nm using a double beam Spectrophotometer (UVIDEC-650, JASCO, Tokyo, Japan). LDL not treated with test substances was used as a control. The results were recorded as the level of conjugated dienes at 6 h and the rate of dienes formation. The concentration of conjugated dienes was calculated by using the molar extinction coefficient 2.8×10^4/M/cm.[23]

Assay of the Formation of Thiobarbituric Acid Reactive Substances (TBARs) Various concentrations (0.001, 0.01, 0.1, 0.25, 1, 5 mg/ml) of roselle were employed in this study. After initiating the oxidation process with $CuSO_4$, the sample mixtures were incubated at 30 °C for 5 h in a water bath and the reaction terminated by adding 50 μl of 100 mM butylated hydroxytoluene (BHT). The following reagents, in the order of 1 ml of 10% trichloroacetic acid (TCA), 0.5 ml of 5 mM disodium ethylenediamine (Na_2EDTA), 1.5 ml of 8% sodium dodecyl sulfate (SDS) and 1.5 ml of 0.6% thiobarbituric acid (TBA), were added into 1 ml of the aliquots taken from the incubation mixture and heated at 80 °C for 1 h. TBARs formation was measured in a spectrofluorimeter at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. 1,1,3,3-Tetraethoxypropane (TEP) was used as astandard.[24] The results were recorded as malondialdehyde (MDA) equivalent content (nmol/mg LDL-protein).

Statistical Analysis All experimental values are presented as means±standard error of mean (S.E.M.). Statistical analysis was performed with analysis of variance (ANOVA) followed by Dunnett's *post hoc* test for multiple comparison among the groups. Difference was considered to be statistically significant if the probability value was less than 0.05 ($p<0.05$).

RESULTS

One hundred micrograms per milliliter of LDL-protein was pre-incubated with 100 μM of vitamin E or various concentrations of roselle (0.001, 0.01, 0.1, 0.25 mg/ml) for 1 h before initiating oxidation with 10 μM $CuSO_4$. Figure 1 shows the effect of roselle on the kinetics of $CuSO_4$-induced LDL oxidation. It clearly shows that $CuSO_4$ dramatically increased oxidation of LDL. The formation of conjugated dienes was inhibited by 100 μM vitamin E. Roselle showed a dose-dependent inhibition of the formation of conjugated dienes. From these kinetic curves, the levels of the conjugated dienes at 6 h and the rates of dienes formation were further determined.

The levels of conjugated dienes at 6 h in $CuSO_4$-induced LDL oxidation of all experiment groups are demonstrated in Fig. 2A. $CuSO_4$ increased the level of the conjugated dienes in LDL about five-fold and was significantly different from control LDL (61.6±9.0 μmol/mg LDL-protein *vs*. 11.6±2.3 μmol/mg LDL-protein, $p<0.05$). Vitamin E completely inhibited the increase in the level of conjugated dienes in $CuSO_4$-induced LDL oxidation (10.7±2.5 μmol/mg LDL-protein, $p<0.05$). Roselle showed a dose-dependent inhibition in increase in the level of conjugated dienes at 6 h. A significant difference was observed at the concentrations 0.1 and 0.25 mg/ml of roselle (22.3±9.0 and 16.1±6.1 μmol/mg LDL-protein, $p<0.05$). The inhibitory effect of 0.25 mg/ml roselle was comparable with that of 100 μM vitamin E. 0.001 and 0.01 mg/ml concentrations of roselle slightly decreased the level of the conjugated dienes at 6 h but was not significantly different from $CuSO_4$-induced group (52.7±9.8 and 40.2±3.7 μmol/mg LDL-protein).

Concomitantly, the addition of $CuSO_4$ in LDL extensively increased the rate of dienes formation, as compared with control LDL (0.122±0.029 μmol/mg LDL-protein/min *vs*. 0.029±0.006 (mol/mg LDL-protein/min, $p<0.05$) (Fig. 2B). Vitamin E 100 μM and roselle 0.25 mg/ml effectively inhibited the rate of dienes formation (0.021±0.008 and 0.028±0.013 μmol/mg LDL-protein/min, $p<0.05$). The concentrations at 0.001, 0.01 and 0.1 mg/ml of roselle slightly decreased the rate of diene formation in LDL in a dose-dependent manner (0.116±0.014, 0.117±0.016 and 0.050±0.017 μmol/mg LDL-protein/min).

TBARs formation, as a marker of lipid peroxidation, was

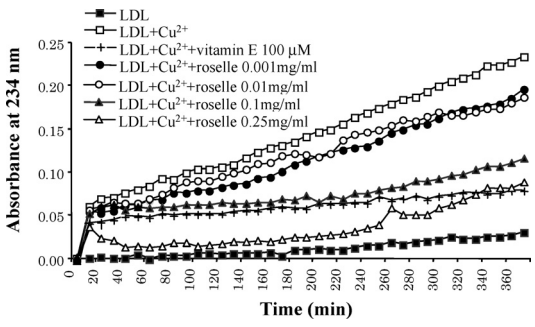

Fig. 1. Effects of Vitamin E and Various Concentrations of Roselle on the Formation of Conjugated Dienes of $CuSO_4$-Induced LDL Oxidation

LDL was pre-incubated with 100 μM of vitamin E or 0.001, 0.01, 0.1 and 0.25 mg/ml of roselle in 10 mM PBS pH 7.4 at 30°C for 1 h before oxidation by adding 10 mM $CuSO_4$. The absorbance at 234 nm was continually measured every 10 min for 6 h. Each point represents the average value from duplicate measurements.

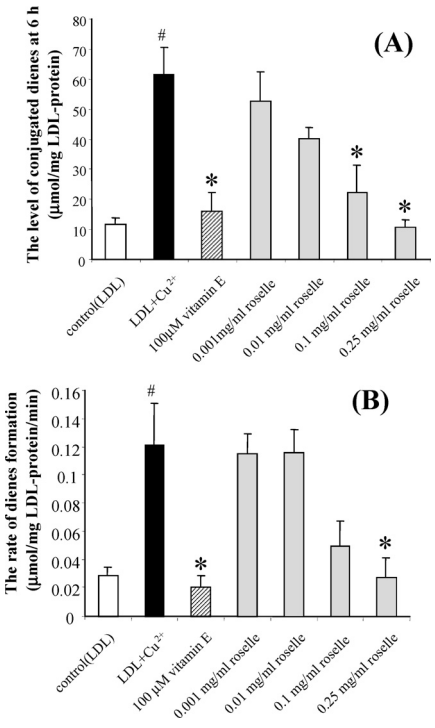

Fig. 2. Effects of Vitamin E and Various Concentrations of Roselle on the Level of Conjugated Dienes at 6 h during the Oxidation of LDL Induced by $CuSO_4$ *in Vitro* (A), the Effect of Roselle on the Rate of Dienes Formation during the Oxidation of LDL Induced by $CuSO_4$ *in Vitro* (B)

The level of conjugated dienes was calculated from the kinetic curve by which using the extinction coefficient of 2.8×10^4/M/cm. The rate of dienes formation was calculated from the slope of the kinetic curve. Data are mean±S.E.M. obtained from 4 independent experiments each performed in duplicated measuring. $*p<0.05$ significant difference from $CuSO_4$-induced group (LDL+$CuSO_4$). $\#p<0.05$ significant difference from control group (LDL).

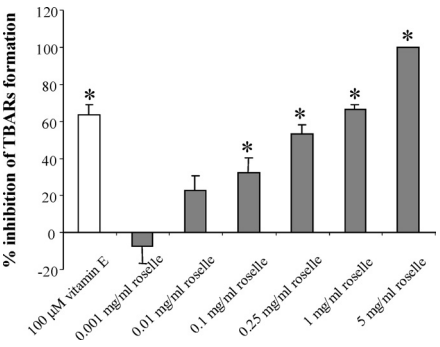

Fig. 3. Inhibitory Effects of Vitamin E and Various Concentrations of Roselle on the Formation of TBARs in $CuSO_4$-Induced Oxidation of LDL

Data are mean±S.E.M. obtained from 3 independent experiments, each performed in duplicate measurements. $*p<0.05$ significant difference from $CuSO_4$-induced group (LDL+$CuSO_4$).

determined and expressed by measurement of MDA equivalent content. Addition of $CuSO_4$ in LDL increased TBARs formation about two-fold and this was significantly increased over control LDL (0.407±0.044 nmol/mg LDL-protein *vs.* 0.189±0.013 nmol/mg LDL-protein, $p<0.05$). Vitamin E 100 μM significantly inhibited TBARs formation about 64% (0.143±0.002 nmol/mg LDL-protein, $p<0.05$). Roselle exhibited a dose-dependent inhibition of TBARs formation. Complete suppression was observed at the concentration of 5 mg/ml of roselle (Fig. 3). 0.1, 0.25 and 1 mg/ml concentrations of roselle significantly inhibited TBARs formation by about 32%, 60% and 66%, respectively (0.276±0.057, 0.162±0.024 and 0.139±0.002 nmol/mg LDL-protein, $p<0.05$). In addition to conjugated dienes formation, our results demonstrate that the inhibitory effect of 0.25 mg/ml of roselle on $CuSO_4$-induced TBARs formation was equivalent to that of 100 μM of vitamin E, while at low concentrations 0.001 and 0.01 mg/ml roselle did not show a significant inhibitory effect on TBARs formation (0.431±0.044 and 0.312±0.053 nmol/mg LDL-protein).

DISCUSSION

Roselle is considered to be a valuable natural substance, the calyx of which has been reported to contain many phytoconstituents such as plant acids (hibiscus acids), phytosterol, flavone derivatives and soluble fibers. Thus, it has been reported to have a variety of biological effects including diuresis, antihypertension and hypocholesterolemia.

Recently, it has been reported that the oxidative modification of LDL (Ox-LDL) is the major factor that stimulates the development of atherosclerosis. Therefore, the major objective of this study was to determine the antioxidant effects of the aqueous extracts from dried calyx of roselle using the *in vitro* model of $CuSO_4$-induced LDL oxidation.

To investigate the potential effect of roselle in the inhibition of $CuSO_4$-induced LDL oxidation, the formation of conjugated dienes and TBARs was determined. Currently, formation of conjugated dienes, measured at 234 nm, is considered the most appropriate marker of LDL oxidation.[20,23] The formation of conjugated dienes represents an early stage in the oxidation process whilst TBARs formation represents a late stage in the oxidation process.[25] Therefore, the formation of conjugated dienes in addition to TBARs formation were measured in this study.

The oxidation of LDL can be divided into 3 phases; lag phase, propagation phase and decomposition phase. The lag phase is the initial interval between the addition of $CuSO_4$ and the beginning of the extensive lipid peroxidation process. During this process, the rate of oxidation is dependent on endogenous antioxidants in LDL. The lag phase is followed by the propagation phase and decomposition phase which result in the formation of aldehydes such as MDA.[26] Vazquez *et al*. reported that rat LDL has a very short lag time (about 15 min) because rat-LDL has a low cholesterol content which indicates a low concentration of total oxidizable substrate in rat-LDL.[27] Base on this, lag time was not determined in this study. Instead, we determined the level of conjugated dienes formed and the rate of dienes formation as indicators of the total amount of conjugated dienes, and the velocity of the oxidation process, respectively.

The present study directly demonstrated directly the protective effect of roselle on LDL oxidation. Pre-incubation of LDL with various concentrations of roselle for 1 h resulted in a dose-dependent antioxidant activity in $CuSO_4$-induced LDL oxidation by inhibiting both the formation of conjugated dienes and TBARs *in vitro*. The results showed that the effective concentration of roselle was about 0.1 mg/ml. The antioxidant effect of roselle is consistent with various experimental studies. Duh and Yen found that a concentration of 1 mg/ml of dried calyx extracts of roselle showed antioxidant activity in the inhibition of TBARs formation in a liposome

model system induced by $FeCl_3$ and ascorbic acid.[17] Tseng *et al*. indicated that concentrations of 0.1 and 0.2 mg/ml of the dried flower extracts of roselle, extracted with chloroform and ethyl acetate, significantly inhibited the formation of malondialdehyde (MDA) induced by *t*-BHP in rat primary hepatocyte cultures.[19] Wang *et al*. suggested that hibiscus anthocyanin, a group of natural pigments occurring in the dried flower of roselle, at concentrations of 0.1 and 0.2 mg/ml significantly decreased the leakage of lactate dehydrogenase and the formation of malondialdehyde (MDA) induced by *t*-BHP in rat primary hepatocyte cultures.[16]

In the present study, we have found a very strong antioxidant effect of roselle through its inhibiting LDL oxidation *in vitro*. A concentration of 0.25 mg/ml of roselle inhibited more than 50% of TBARs formation induced by $CuSO_4$. Moreover, a concentration of 5 mg/ml of roselle showed a remarkable antioxidant effect on $CuSO_4$-induced LDL oxidation through its complete inhibition of TBARs formation. In addition, its antioxidant effect at this concentration was more effective than 100μM of vitamin E which was used as the standard control under the same conditions. We therefore clearly demonstrate that roselle is a potent antioxidant in the protection of LDL oxidation induced by $CuSO_4$ *in vitro*.

A number of mechanisms by which antioxidants protect LDL from oxidation may be considered. Firstly, they may function as chelators which inactivate Cu^{2+} and other ions involved in the initiation of free radicals *in vitro*. Secondly, they may function as chain breaking substances which directly prevent the formation of free radicals mediated by Cu^{2+}. Finally, they may function as a hydrogen atom donor to α-tocopherol radicals and other antioxidants in LDL particles.[20] The molecular mechanisms by which roselle inhibits LDL oxidation *in vitro* were not determined in this study. However at beginning of this study, we determined the PCA content in the dried calyx of roselle was determined. It is possible that the antioxidant effect is derived from PCA within the extracts. Laranjinha *et al*. evaluated that PCA retards lipid peroxidation of LDL initiated by forming complex reactive species with peroxyl radicals.[28] PCA possesses a catechol group and the laterol COOH group is ionized at the experimental pH 7.4. Since COO^- efficiently releases electrons, a stronger antioxidant activity is to be expected. Tseng *et al*. reported that hibiscus PCA was a protective agent against oxidative damage induced by *t*-BHP in primary cultures of rat hepatocytes.[18] The inhibitory effect on MDA formation was observed at the concentrations 0.05 mg/ml and 0.1 mg/ml. These results were comparable with our study.

Thus, it may be speculated that the antioxidant effect of roselle in the protection of LDL from oxidative modification *in vitro* may result from either PCA or other polyphenolic compounds in the roselle. Further studies should be conducted to clarify the exact mechanism(s) of action.

In conclusion, our results showed that the aqueous extracts from dried calyx of *Hibiscus sabdariffa* Linn. possess a potent, dose-dependent antioxidant effect in the protection of LDL oxidation induced by $CuSO_4$ *in vitro*. We are now investigating the antioxidant and antiatherosclerotic effects of roselle *in vivo* in rats.

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