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Inhibition of Low-Density Lipoprotein Oxidation and Up-Regulation of Low-Density Lipoprotein Receptor in HepG2 Cells by Tropical Plant Extracts

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Twelve edible plant extracts rich in polyphenols were screened for their potential to inhibit oxidation of low-density lipoprotein (LDL) in vitro and to modulate LDL receptor (LDLr) activity in cultured HepG2 cells. The antioxidant activity (inhibition of LDL oxidation) was determined by measuring the formation of conjugated dienes (lag time) and thiobarbituric acid reagent substances (TBARS). Betel leaf (94%), cashew shoot (63%), Japanese mint (52%), semambu leaf (50%), palm frond (41%), sweet potato shoot, chilli fruit, papaya shoot, roselle calyx, and maman showed significantly increased lag time (>55 min, $P < 0.05$) and inhibition of TBARS formation ($P < 0.05$) compared to control. LDLr was significantly up-regulated ($P < 0.05$) by Japanese mint (67%), semambu (51%), cashew (50%), and noni (49%). Except for noni and betel leaf, most plant extracts studied demonstrated a positive association between antioxidant activity and the ability to up-regulate LDL receptor. Findings suggest that reported protective actions of plant polyphenols on lipoprotein metabolism might be exerted at different biochemical mechanisms.

KEYWORDS: LDL oxidation; LDL receptor; plant extracts; TBARS; HepG2 cells

INTRODUCTION

The specific health benefits of fruits and vegetables have been known for centuries, and more recent epidemiological studies clearly demonstrate that plant-based foods protect against several chronic disease conditions including cardiovascular disease (*1*). Elevated plasma low-density lipoprotein (LDL) cholesterol is a well-established risk factor for atherosclerosis. The LDL receptor (LDLr), which binds LDL in the circulation, plays an important role in maintaining the level of cholesterol (*2*). Liver LDLr predominantly determines the uptake of LDL cholesterol and regulates the plasma LDL concentration.

LDL particles are susceptible to oxidation in the presence of free radicals and metal transition ions such as copper. Modified oxidized LDL particles are atherogenic because they have a higher tendency to enter and accumulate in the arterial wall, causing endothelial damage (*3*, *4*). This modified LDL is no longer recognized by the LDLr. Instead, it is taken up by the scavenger receptor on the monocytes−macrophages (*5*), forming foam cells, the key component of the fatty streak lesions of atherosclerosis. Therefore, dietary strategies to prevent oxidation of LDL and up-regulation of LDLr activity are likely to result in beneficial outcomes.

It is now recognized that cardiovascular protective effects of fruits and vegetables may at least in part be mediated via their hypocholesterolemic and antioxidant activities (*6*−*9*). It is likely that flavonoids, which are found abundantly in edible plants, contribute to these protective effects (*10*) as demonstrated by green tea and grape products that inhibit LDL oxidation in vitro (*11*, *12*). Although there exists a substantial amount of information regarding inhibition of LDL oxidation by phytochemicals (*6*−*9*, *11*−*13*), relatively few studies have investigated the potential of plant constituents to modulate LDLr (*14*−*16*). Furthermore, it is not clearly established whether any association exists between antioxidant activity and the ability to up-regulate LDLr activity by phytochemicals.

In the present study, polyphenol-rich extracts of 12 edible plants were examined for their potential to inhibit LDL oxidation (antioxidant activity) and the modulation (up- or down-regulate) of LDLr activity in HepG2 cells. Most of the plants selected are commonly found in the Malaysian diet, and some have perceived medicinal values.

EXPERIMENTAL METHODS

Preparation of Test Extracts. The 12 edible tropical plants examined in this study were betel leaf (*Piper betle*), sweet potato shoot (*Ipomoea batatas*), palm frond (*Elaeis guineensis*), noni leaf (*Morinda*

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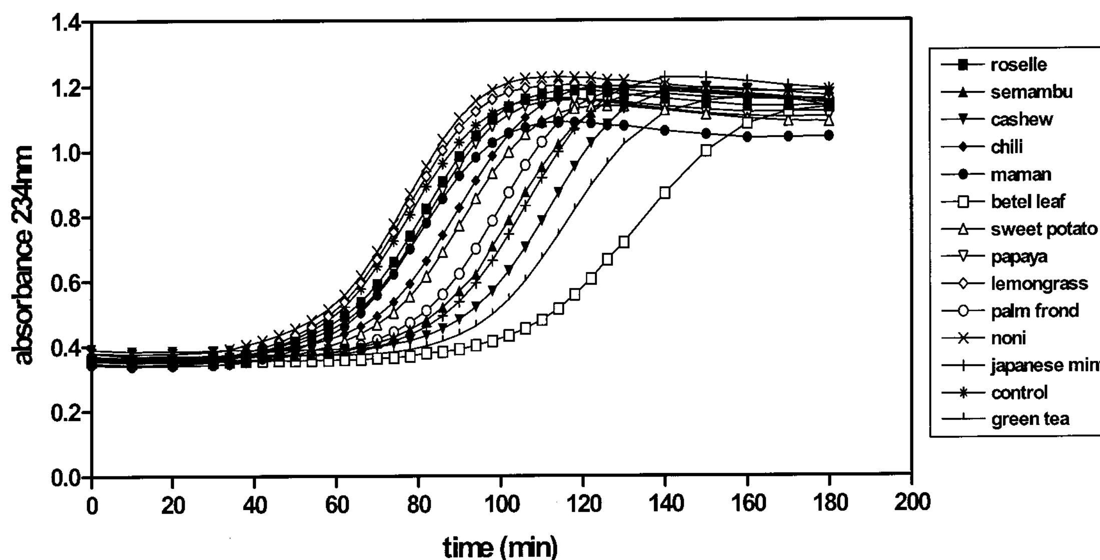

Figure 1. Effects of various plant extracts on conjugated diene formation of copper-mediated LDL oxidation. Fifty milligrams of LDL protein/mL was incubated with 5 μmol/L copper sulfate (5 μmol/L) in the presence of test extracts (12.5 μg/mL) in PBS at 37 °C for 3 h, and conjugated dienes were monitored continuously at 234 nm as described under Experimental Methods. Control (buffer); positive control (green tea). Assays were performed in duplicate, and mean ($n = 3$) values are shown. SEM values have been omitted for clarity of presentation.

citrifolia), Japanese mint (*Mentha arvensis*), maman leaf (*Gynandropsis gynandra*), cashew shoots (*Anacardium occidentale*), papaya shoots (*Carica papaya*), chilli fruits (*Capsicum frutescens*), semambu leaf (*Calamus scipronum*), roselle calyx (*Hibiscus sabdarifa*), and lemongrass (*Cymbopogon citrates*). The leaves, stems, and shoots were rinsed, dried, cut into smaller pieces, and freeze-dried. The dried material was then ground into powder and extracted three times with methanol (10% w/v). The pooled organic phase was filtered (Whatman No. 1), vacuum-dried at 40 °C, flushed with nitrogen, and stored at −80 °C protected from light and air.

LDL Oxidation Assay. *LDL Preparation.* Blood was collected in tubes containing EDTA (1 mg/mL) from overnight-fasted healthy adult volunteers. Plasma was isolated by centrifugation (3000*g* for 10 min) and stored at −80 °C for no longer than 8 weeks. Storage of plasma for this period does not alter the LDL oxidation rate and lag time for conjugated diene formation (*17*). Prior to the oxidation experiments, LDL was isolated from plasma by a single-step ultracentrifugation method as described by Abbey et al. (*18*). Protein was determined according to the Lowry method (*19*).

Conjugated Diene Formation and Thiobarbituric Acid Reactive Substance (TBARS) Assay. Conjugated diene formation and the TBARS assay were used to evaluate the extent of LDL oxidation. Conjugated diene formation was determined as described by Kerry and Abbey (*20*). In brief, following isolation of LDL and subsequent dialysis to remove EDTA, 50 mg of LDL protein/mL was incubated with 5 μmol/L copper sulfate (5 μmol/L) in the presence of test extracts (12.5 μg/mL) in phosphate-buffered saline (PBS) at 37 °C for 3 h. Control incubations were carried out using PBS. The concentration of test extracts was selected from preliminary studies. The formation of conjugated dienes was monitored at 2 min intervals at 234 nm (Beckman DU-65). The lag phase (expressed in minutes) is the interval between the addition of $CuSO_4$ and the beginning of the propagation phase. Lag time was measured on the basis of the intercept between the baseline and tangent to the propagation phase (*21*).

Malondialdehyde (MDA) formation in copper-mediated LDL oxidation was determined by TBARS assay according to the method of Beuge and Aust (*22*). Within 3 h following the oxidation, 100 μL samples were taken hourly into 1.5 mL tubes after being stopped by BHT and EDTA. After sample collection, TBARS reagent (200 mL) (15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N hydrochloric acid) was added and mixed vigorously. The mixture was incubated in boiling water for 15 min to develop the MDA−TBA adducts followed by centrifugation. The supernatant (200 μL) of MDA−TBA adducts was measured at 535 nm on a Cobas Bio autoanalyzer (Roche Diagnostica, Nutley, NJ). TBARS formation was expressed as nanomoles of MDA per milligram of protein.

LDL Receptor Assay. *HepG2 Cell Culture.* HepG2 cells were grown in monolayer cultures to near confluency in 25 cm^2 flasks with 5 mL of Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) at 37 °C with 5% CO_2 (*23*). Cells were then incubated for 24 h in flasks with 5 mL of DMEM/FCS containing 50 μL (10 mg/mL) of each test extract in triplicate. Methanol served as a control, and an organic extract of green tea (10 mg/mL) was used as the positive control.

LDLr Binding Activity. Following incubation, cells from each flask were harvested and resuspended in PBS, and the protein content was measured (*19*). The specific LDLr binding activity was measured as previously described (*24*). The intact cells (100 μg of protein) were incubated for 1 h at room temperature with LDL−gold conjugates (20 μg of protein/mL) in the absence or presence of 20 mM EDTA to determine total and nonspecific binding, respectively. After 1 h, cells were pelleted by centrifugation, washed, and resuspended in 4% (w/v) gum arabic, and a silver enhancement IntenSE BL kit solution (Amersham, Sydney, Australia) was then added. The silver enhancement reaction and absorbance measurements (500 nm) were carried out using the Cobas Bio autoanalyzer (Roche Diagnostica). The amount of LDL bound was expressed as nanograms of LDL per milligram of cell protein. Duplicate determinations were made for both total and nonspecific binding, and specific binding (total minus nonspecific binding) was taken as the measure of LDLr binding activity. Up-regulation of the LDLr is expressed as the percentage of increased binding activity compared to control.

Statistics. Results are expressed as mean ± SEM. Statistical evaluation was performed using a two-tailed Student's *t* test, comparing the appropriate control with different plant extracts as indicated. The value of $P < 0.05$ was considered to be significant.

RESULTS

LDL Oxidation. All extracts except lemongrass and noni significantly ($P < 0.05$) delayed the oxidation of LDL as evident by increased lag time compared to control (**Figure 1**). The formation of conjugated dienes agreed with the trend proposed by Esterbauer et al. (*21*), with betel leaf having the longest inhibitory effect on LDL oxidation in the initiation phase of the assay compared to green tea (**Figure 1**).

Table 1. Inhibition of Copper-Mediated Oxidation of LDL by Plant Extracts[a]

sample	lag time (min)	% inhibition
control	55.2 ± 0.8	0
green tea	97.0 ± 2.2[a]	76
betel leaves	106.9 ± 5.8[a]	94
cashew	89.9 ± 3.3[a]	63
mints	84.0 ± 2.0[a]	52
semambu	82.5 ± 1.4[a]	50
palm frond	77.9 ± 0.7[a]	41
sweet potato	69.0 ± 1.8[a]	25
chilli	67.7 ± 1.8[a]	23
papaya	59.8 ± 0.8[a]	8
roselle	59.2 ± 1.3[a]	7
maman	58.3 ± 0.9[a]	6
noni	54.8 ± 1.3	−1
lemongrass	54.8 ± 1.7	−1

[a] Assays were done in triplicate, and the values given are the means ± SEM of $n = 3$ separate determinations. Control incubations were carried out in the presence of phosphate-buffered saline. Superscripts indicate significant difference compared to control at $P < 0.05$ (Student's t test).

Table 2. Plant Extract-Induced Inhibition of TBARS Formation during Cu^{2+}-Mediated Oxidation of LDL[a]

sample	MDA (1 h)	% inhibition (TBARS)	MDA (2 h)	% inhibition (TBARS)
control	31.3 ± 11.7	0	88.1 ± 3.6	0
green tea	3.7 ± 1.8[a]	88	52.7 ± 14.0[a]	40
betel leaves	3.0 ± 0.3[a]	90	32.7 ± 7.4[b]	63
cashew	2.2 ± 1.0[a]	93	34.7± 14.3[b]	60
mints	7.0 ± 3.3	77	74.0 ± 7.3	16
semambu	7.3 ± 3.0	76	68.4 ± 10.5	22
palm frond	8.0 ± 3.2	74	83.3 ± 4.2	5
sweet potato	16.7 ± 5.0	46	85.1 ± 5.0	3
chilli	16.5 ± 6.9	47	78.4 ± 7.5	11
papaya	24.9 ± 9.4	20	86.8 ± 3.9	1
roselle	19.6 ± 6.0	37	85.0 ± 3.3	3
maman	29.6 ± 11.0	5	85.5 ± 3.5	3
noni	29.5 ± 11.1	5	83.7 ± 5.5	5
lemongrass	24.9 ± 8.9	20	88.5 ± 2.0	0

[a] LDL (50 mg of LDL protein/mL) was incubated in the presence of $CuSO_4$ (5 μmol/L) for 3 h as described under Experimental Methods. TBARS formation was expressed as nmol of MDA/mg of protein, and the values given are the means ± SEM (triplicate determinations; three separate experiments). Superscripts a and b indicate significant difference compared to control at $P < 0.05$ and $P < 0.01$, respectively (Student's t test). For clarity results at 3 h are not shown.

As shown in **Table 1**, inhibitory action varied greatly (6−94%) for the different extracts, with the highest being observed for betel leaf (94%) followed by cashew (63%), Japanese mint (52%), semambu (50%), and palm frond (41%). Green tea, the positive control, significantly inhibited oxidation ($P < 0.05$, 78%).

Time-dependent TBARS formation is shown in **Table 2**. After 1 h, green tea (88%), cashew (93%), and betel leaf (90%) resulted in significantly lower TBARS formation than the buffer control ($P < 0.05$). This trend was maintained at 2 h, with the buffer control recording greater TBARS than green tea (35%, $P < 0.05$), cashew (60%, $P < 0.01$), and betel leaf (63%, $P < 0.01$) extracts. In contrast, there was no significant difference observed at 3 h. A positive correlation between TBARS and conjugated diene formations was observed at 1 h ($R^2 = 0.82$) and 2 h ($R^2 = 0.70$).

LDLr. Figure 2 shows that the addition of green tea extract (positive control) resulted in increased (35%) LDLr binding activity ($P < 0.05$ vs buffer control). Among the extracts tested, noni (49%), Japanese mint (67%), semambu (51%), and cashew

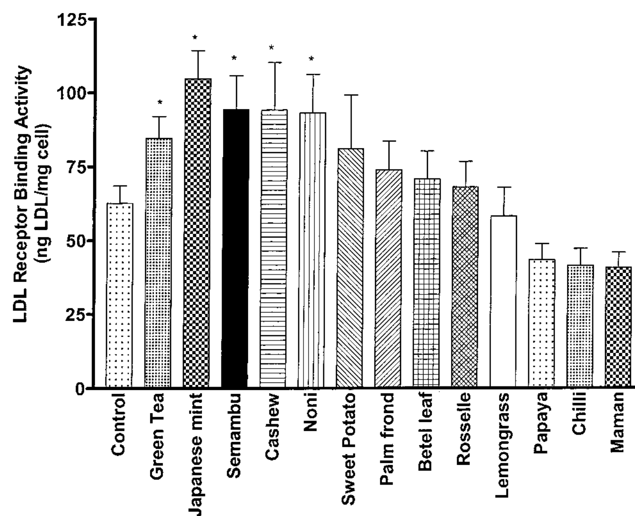

Figure 2. Effect of different plant extracts on LDLr binding activity in cultured HepG2 cells. The LDLr binding activity was measured as the calcium-dependent binding of LDL–gold to intact cells as described under Experimental Methods. The values are mean ± SEM of triplicate cell incubations ($n = 3$ experiments). Asterisks indicate significant difference compared to control (vehicle) at $P < 0.05$. Positive control (green tea extract).

(50%) increased LDLr binding activity ($P < 0.05$) compared to control. These extracts up-regulated the LDLr in HepG2 cells more than the positive control (green tea).

DISCUSSION

Most plant extracts tested in the present study significantly increased LDL resistance to oxidation in vitro. It was found that when LDL was subjected to copper-mediated oxidation, the presence of test extracts increased the lag phase of conjugated diene formation (**Figure 1**) and decreased the TBARS formation (**Table 2**). Similarly, LDLr activity was significantly up-regulated by several plant extracts (**Figure 2**). Although inhibition of LDL oxidation by a range of different plant sources has been widely reported (*6−9*, *11−13*), relatively few studies have focused on the effects of phytochemicals on LDLr activity (*14−16*). The antioxidant and LDLr modulation effects have not been described previously for the plant extracts used in this study.

The inhibition of copper-mediated LDL oxidation by phytochemicals including flavonoids and α-tocopherol has been well established (*25*, *26*). In the present study, determination of the composition of extracts was not attempted. However, on the basis of the values reported previously for several of the plant materials used in this study (*27*, *28*), the inhibition of LDL oxidation does not appear to show any direct association with the major flavonoids (myricetin, quercetin, kaempferol, luteolin, and apigenin) or α-tocopherol contents. For instance, whereas semambu with a relatively high inhibition of LDL oxidation (50%) has been found to be enriched in flavonoids and α-tocopherol (2041 and 320 mg/kg of dry weight, respectively), betel leaf, which exhibited the highest inhibitory action (94%), contained only a trace amount of α-tocopherol and no detectable levels of myricetin, quercetin, kaempferol, luteolin, and apigenin (*27*, *28*). Betel leaf, on the other hand, has been found to have higher amounts of carotene and ascorbic acid (*29*), both of which are strong antioxidants. These analytical data suggest that the activity of betel leaf in inhibiting LDL oxidation observed in the present study could be due to these latter antioxidant

compounds and/or mediated via different polyphenol compounds not quantified previously (*27*).

The importance and clinical relevance of the LDLr in normal regulation of plasma LDL cholesterol concentration has been documented (*30*). However, there is relatively little published work on the ability of plant constituents to modulate LDLr activity specifically, except for green tea polyphenols and antioxidant vitamins (*15*, *16*). A positive relationship between antioxidant activity and up-regulation of LDLr in HepG2 cells has been previously reported for vitamins A and C, β-carotene, and green tea catechins (*15*). The present findings are in agreement with the earlier study; with the exception of noni and betel leaf, a similar association was observed ($R^2 = 0.55$). The noni extract did not inhibit LDL oxidation but showed a relatively good ability to up-regulate the LDLr. In contrast, betel leaf, which significantly inhibited oxidation of LDL, failed to upregulate the LDLr ($P > 0.05$). The possibility exists that biological effects at the cellular level of betel leaf extract may be concentration dependent, as has been reported for vitamin E (*15*). However, no dose−response relationship was investigated in the present study. The mechanisms by which different plant extracts inhibited LDL oxidation and up-regulated the LDLr activity remain to be investigated. In this context it is noteworthy that green tea catechins have recently been reported to up-regulate LDLr through the sterol-regulated element binding protein (SREBP) in HepG2 cells (*16*).

Up-regulation of the hepatic LDLr leads to accelerated clearance of LDL from the circulation (*31*), reducing its exposure to possible oxidative modification and thus lowering the risk potential for atherosclerosis (*3*). Findings in this study further support the notion that edible plants possess specific health benefits that may be effective in combating the pathogenesis of chronic diseases. A greater understanding of the mechanism(s) of action of different plant constituents may have implications for both dietary prevention strategies and their potential use as nutraceuticals to promote optimal cardiovascular health.

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