Endothelial Cytoprotection from Oxidized LDL by Some Crude Melanesian Plant Extracts is Not Related to Their Antioxidant Capacity

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ABSTRACT: Habitual consumption of some Melanesian medicinal and food plants may influence atherosclerosis development via their antioxidant capacity at the endothelial level. Areca nut (AN; *Areca catechu*), piper inflorescence (PBI; *Piper betle*), betel quid (BQ), guava buds (GB; *Psidium guajava*), the leaves (NL), juice (NJ), fruit (NF), and root (NR) of noni (*Morinda citrifolia*), the propagules of raw (MBR), and cooked (MBC) mangrove (*Bruguiera gymnorrhiza*) were evaluated for their ability to scavenge the 1,1-diphenyl-2-picryl-hydrazyle (DPPH) radical, to protect human low-density lipoprotein (LDL) from Cu^{2+}-catalyzed oxidation and to protect cultured bovine aortal endothelial cells (BAEC) from oxidized LDL (oxLDL)-induced cytotoxicity. Polyphenol-rich extracts AN, PBI, and BQ were potent DPPH scavengers, having similar activity to quercetin and able to protect LDL from oxidation in a dose-dependent manner at concentrations higher than 10 μg/mL, but were pro-oxidants at lower concentrations. These extracts were cytotoxic to BAEC at concentrations above 10 μg/mL and were unable to prevent oxLDL endotheliopathy. GB and NR at 10 μg/mL displayed both the ability to delay LDL oxidation and prevent oxLDL cytotoxicity, although the latter lacked the ability to scavenge the DPPH radical. At higher concentrations, however, both were cytotoxic in themselves. The remaining noni extracts NF, NJ, NL, and both mangrove extracts MBC and MBR were unable to protect LDL from oxidation at all tested concentrations, but were effective cytoprotective agents at 50 μg/mL. All extracts were able to prevent an oxLDL-mediated increase in intracellular aldehyde generation but had little effect on extracellular peroxidation as measured by thiobarbituric acid reactive substances (TBARS). On the basis of this model system, we conclude that the antioxidant benefits of AN, PBI, and BQ may be offset by their enhancement of their cytotoxic effects of oxLDL toward BAEC, whereas GB and low concentrations of noni and mangrove may be considered antiatherogenic. The discrepancies between our in vitro and cellular culture experiments emphasize the importance of experimental conditions in evaluating the antioxidant potential of crude plant extracts.
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INTRODUCTION

Differential rates of metabolic disease in Melanesia are underlined by contrasts in dietary and medicinal plant use. In an epidemiological investigation of traditional dietary and medicinal plant use patterns and cardiovascular health in coastal Papua New Guinea, plants that were routinely used in one setting and absent in others were collected for laboratory testing. Betel quid (BQ), a socioculturally important masticatory consisting of areca nut (AN; *Areca catechu*), the inflorescence of *Piper betle* (PBI), and slaked lime is chewed by the vast majority of the population as a psychoactive stimulant. An infusion of guava buds (GB; *Psidium guajava*) was consumed habitually in a

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few coastal villages as a gastrointestinal and circulatory tonic. Noni (*Morinda citrifolia*), perhaps the most popular medicinal in the Pacific region, was used by most coastal communities as a tonic. Traditionally, the fruit and its juice were eaten for the treatment of asthma, tuberculosis, and diarrhea. Leaves were most often used externally to treat wounds and sores but occasionally consumed for mouth sores and stomach aches. The root tea was ingested as a treatment for urinary disorders. The hypocotyls of wild mangrove (*Bruguiera gymnorhiza*) form the primary energy staple of a small number of remote rural villages.

Growing evidence supports an association between the consumption of antioxidant-rich foods and reduced risk of atherosclerosis [1]. A common hypothesis concerns the inhibition of low-density lipoprotein (LDL) oxidation, a key step that would otherwise initiate a pathological response resulting in chemotaxic migration of monocytes to the subintima of blood vessels and formation of characteristic macrophage-foam cells that are inherent in atherosclerotic lesions [2]. Other biological effects of oxidized LDL (oxLDL) include upregulation of endothelial adhesion molecules, expression and release of growth factors and cytokines, and proliferation of smooth muscle cells [3]. Oxidized LDL is further atherogenic in promoting vascular dysfunction by directly exerting cytotoxicity. Concomitant intracellular increases in thiobarbituric acid reactive substances (TBARS) and Ca^{2+} are also associated with oxLDL cytotoxicity toward cultured endothelial cells, resulting in cell death [4].

Several dietary factors such as α-tocopherol [5] and plant phenolics [6] are able to protect endothelial cells from oxLDL cytotoxicity through a nonantioxidant mechanism involving inhibition of intracellular signaling events. However, other phenolic compounds, such as quercetin, display conditional pro-oxidant activity and cytotoxicity, highlighting the importance of a phenol's molecular structure and experimental conditions [7].

Concomitant with a loss of traditional dietary and medicinal practices in developing nations, there is a dramatic increase in the incidence of cardiovascular disease. The need to identify protective elements in indigenous systems as a culturally sensitive health-promotion strategy prompted us to examine the antioxidant properties of selected plants in relation to cardiovascular health, irrespective of their traditional use. In this study, crude MeOH plant extracts were screened for general free radical scavenging activity using the stable radical 1,1-diphenyl-2-picryl-hydrazyle (DPPH). Various extract concentrations were then tested in vitro for their ability to affect lag time before the appearance of conjugated dienes and production of TBARS resulting from Cu^{2+}-induced LDL oxidation. Lastly, extracts were incubated for 24 h with bovine aortic endothelial cells (BAEC) to determine maximal nontoxic concentrations by measuring extracellular lactate dehydrogenase (LDH) release, after which oxLDL was added and cytotoxicity monitored up to 6 h. Extra- and intracellular generation of aldehydes resulting from lipid peroxidation was measured as TBARS in the medium and lysate, respectively, to assess an extract's ability to confer cellular resistance to oxLDL-induced oxidative stress. Although biomechanisms were not elucidated, possible bioactive components that explain association patterns between the antioxidative and cytoprotective properties of plant extracts are discussed.

MATERIALS AND METHODS

Plant Collection and Extract Preparation

Fresh plant material was collected in Papua New Guinea (Table 1), oven-dried at 100°C for 1 h and brought back to Canada for methanolic extraction as described in Owen and Johns [8]. Voucher specimens were deposited at the University of Papua New Guinea and McGill University herbarium. Betel quid (BQ) was prepared by combining approximately 66.6% *A. catechu*, 26.6% *P. betel*, and 6.6% calcium hydroxide before extraction. Cooked mangrove bean (MBC) was prepared according to traditional methods: thin slices were soaked for 1 h and boiled in two changes of sea water. Noni juice (NJ) was purchased at a local health food store (Flora Manufacturing & Distributing Ltd, Burnaby, BC).

Total and Water-Soluble Phenol Concentration

Total and water-soluble phenol concentrations were determined using the Folin-Ciocalteau method as described in Owen and Johns [9]. Phenol concentration was obtained from a standard curve of tannin and expressed as milligram tannic acid equivalents (TAE)/g extract.

Free Radical Scavenging Activity

Methanol crude extracts were screened for nonspecific free radical scavenging activity using the 1,1-diphenyl-2-picryl-hydrazyle (DPPH) radical as described in Owen and Johns [8]. The efficiency concentration at 50% (EC_{50}) was calculated by determining the extract concentration required to quench all DPPH radicals in solution, determined by observing no further change in absorbance with increased concentration,

TABLE 1. Characteristics of Plants, Extract Yield, and Content of Total and Water-Soluble Phenols

Family, Species	*Plant Part*	*English, Local Name*	*Extract Yield (%)*[a]	*Voucher No.*	*Total Phenols (mg TAE/g extract)*	*Water Soluble Phenols (mg TAE/g extract)*
Arecaceae						
Areca catechu L.	Nut	Betelnut, Buai	24.35	KAL23	17.83 ± 1.78	10.72 ± 0.71
Myrtaceae						
Psidium guajava L.	Bud	Guava, Tuava	18.35	KAL01	11.52 ± 0.77	9.40 ± 0.18
Piperaceae						
Piper betle L.	Inflorescence	Pepper, Daka	12.50	KAL24	11.48 ± 0.37	7.98 ± 1.32
Rhizophoraceae						
Bruguiera gymnorrhiza (L.) Lam.	Cooked hypocotyls	Mangrove bean, Kavela	2.1	WAN01	1.35 ± 0.94	0.68 ± 0.08
	Raw hypocotyls		23.5		6.38 ± 1.12	4.23 ± 0.12
Rubiaceae						
Morinda citrifolia L.	Fruit	Noni, Nono	29.35	KAL03	1.36 ± 0.36	0.45 ± 0.00
	Commercial juice				0.64 ± 0.26	0.26 ± 0.01
	Leaf		23.30		2.06 ± 0.99	1.33 ± 0.05
	Root		17.25		1.82 ± 0.07	0.30 ± 0.06
Betel quid			13.25		9.97 ± 0.70	6.15 ± 0.20

TAE: tannic acid equivalents.
[a] Extract yield = (g extract/g dried plant material) $\times$ 100.

and dividing it in half. Results were standardized to account for interference from pigments. Ascorbic acid, quercetin and epicatechin (Sigma Chem. Co., St. Louis, MO) were used as standards.

Low-Density Lipoprotein Preparation

Human LDL in solution containing 0.15 NaCl and 0.01% EDTA (Intracel, Frederick, MD) was diluted in 4 mL PBS and passed through a Sephadex PD-10 column (Pharmacia Biotech, Uppsala, Sweden) to remove most of the NaCl and EDTA. Lipoprotein concentration was estimated with a Sigma Diagnostics protein assay kit (Sigma Chem. Co.) using bovine serum albumin as a standard. For cell culture, extensive modification of LDL (malondialdehyde-rich with high-lipid peroxide content) was obtained by overnight (8 h) incubation with 15 μM $CuSO_4$ at 37°C, sterilized by filtration (0.2 μm Millipore membrane).

Conjugated Dienes Formation

Lag time was measured using the methods of Esterbauer et al. [10]. In a UV-transparent microtiter plate, 100 μg/mL LDL protein, 5 μM $CuSO_4$ dissolved in PBS, and 1, 5, 10, and 25 μg/mL plant extract dissolved in 50% MeOH were mixed to a final volume of 200 μL and absorbance was continuously monitored at 234 nm using a uQuant™ universal microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). Corresponding concentrations for ascorbic acid (5.67, 28.39, 56.78, and 141.95 μM) and Trolox® (4, 19.98, 39.95, and 99.89 μM) were used as positive controls (data not shown). Results are expressed as percent increase or decrease in lag time relative to the lag time of Cu^{2+}-oxidized LDL (oxLDL) incubated without extract or standard.

Measurement of Thiobarbituric Acid Reactive Substances

Following the methods of Sobal et al. [11], 100 μg/mL LDL protein, 5 μM $CuSO_4$ dissolved in PBS, and 1, 5, 10, and 25 μg/mL plant extract dissolved in 50% MeOH were made up to 1 mL with PBS and left to incubate at room temperature. At 0, 90, 180, and 360 min, 120 μL was removed in duplicate, placed on ice for 5 min and 10 μL each of 10 μM BHT and 400 μM EDTA added to halt oxidation. Thereafter, 50 μL 50% (w/v) trichloroacetic acid (Sigma Chem. Co.) and 75 μL 1.3% (w/v) thiobarbituric acid were added and the reaction mixture was incubated at 60°C for 40 min. Tubes were then cooled on ice for 5 min, followed by centrifugation at 2000×g for 10 min, after which 200 μL of the supernatant was transferred to a microtiter plate and fluorescence (excitation wavelength 510 nm/emission wavelength 553 nm) recorded using a Wallac Victor^2 multilabel counter (PerkinElmer Inc., Waltham, MA). Concentration of TBARS was obtained using a 1,1,3,3-tetraethoxypropane (malondialdehyde, MDA) standard curve and results expressed as percent (%) increase or decrease relative to the TBARS levels generated from Cu^{2+}-oxidized LDL (oxLDL) incubated without extract or standard. Ascorbic acid and Trolox were used as positive controls (data not shown).

Cell Preparation

Bovine aortic endothelial cells were maintained in 12-well plates containing Dulbelco's modified Eagle's medium (DMEM; Life Tech. Inc., Burlington, ON) with 0.3 g/L glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS), 20,000 U/L penicillin and 20,000 μg/L streptomycin in a humidified 37°C atmosphere containing 5% CO_2 and 95% air. Cells had fewer than five passages and all experiments were performed two-day postconfluence.

Cytotoxicity of Plant Extracts and oxLDL

Cytotoxicity expressed as percent (%)LDH release ([extracellular LDH]/[intracellular LDH]*100) was measured using a commercially available kit (Sigma Chem Co.). Plant extracts and the positive control BHT were dissolved in DMSO and added to wells at 0.01% final volume. DMSO was tested alone to ensure that any activity was not due to the vehicle. Prior to the addition of oxLDL, plant extracts, 10 μM BHT, and DMSO were incubated for 24 h to allow any possible incorporation into BAEC. The highest concentration of extract tolerated by BAEC without incurring any significant increases in LDH release over 24 h was found to be 10 μg/mL for AN, PBI, BQ, GB, and NR, and 50 μg/mL for the remaining extracts. This maximal nontoxic concentration was used for our cell culture experiments. Following 24-h incubation with plants extract or BHT, 100 μg oxLDL was added to the medium and LDH release was measured in the supernatant and lysate at 0, 180, and 360 min. Native LDL was measured in the absence of test samples. Results are expressed as percent (%) LDH increase relative to normal baseline levels of cells grown in the absence of oxLDL and plant extracts.

Measurement of Cellular TBARS

Extra- and intracellular aldehyde generation were determined as TBARS in the medium and lysate, respectively. At 0, 180, and 360 min, plates were placed on ice for 5 min and the medium immediately collected and separated into aliquots of 120 μL to which 10 μL of 10 μM BHT and 400 μM EDTA were added to halt oxidation. For lysates, cold PBS was added and cells were scraped, sonicated for 10 min, centrifuged at $250\times g$ for 5 min and supernatant collected and treated as per the protocol for the medium. Determination of TBARS was as above. Results are expressed as percent (%) increase or decrease in TBARS concentration relative to normal baseline levels of cells incubated in the absence of oxLDL and plant extracts.

Statistics

Results are expressed as mean ± SEM from at least three independent experiments performed in duplicate. A one-tailed ANOVA with post hoc Tukey's was used to test significant differences between samples for DPPH. Differences in lag time, in vitro and cellular TBARS, and cellular LDH were assessed using Student's *t*-test. Spearman's correlation was used to assess associations between assays. Significance was established at $p < 0.05$.

RESULTS

DPPH-Scavenging Assay

Several phenolic compounds are known antioxidants that are able to scavenge free radicals. In this assay, plant extracts that had a higher content of total and water-soluble phenols (Table 1) were better scavengers of DPPH than those with little or no phenolic content. Thus, BQ and its constituents AN and PBI, which had a phenol content of 9.97 ± 0.70, 17.83 ± 1.78, and 11.48 ± 0.37 mg TAE/g extract, respectively, were relatively strong scavengers of DPPH, with activity comparable to that of the antioxidant flavonoid quercetin (Figure 1). Guava bud (GB) extract (11.52 g TAE/g extract) demonstrated the highest scavenging capacity of the extracts tested, with an $EC_{50} = 8.05 \pm 0.49$ μg/mL. Raw mangrove bean (MBR) extract also exhibited significant DPPH-scavenging activity ($EC_{50} = 12.22 \pm 0.50$), but removal of tannins and phenols via traditional processing (approximately 80% of total phenols in MBR were lost when preparing MBC) caused a complete loss of activity. Similarly, none

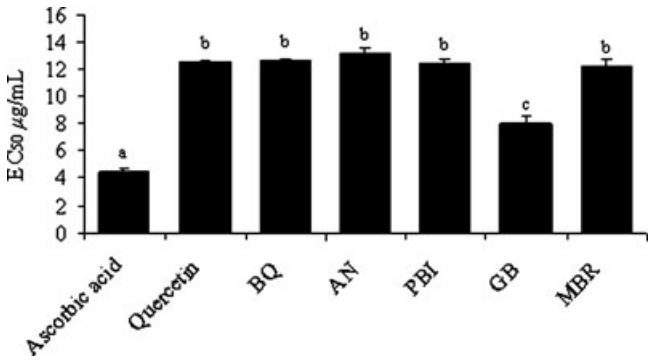

FIGURE 1. Free radical scavenging activity of selected crude plant extracts using the stable radical 1,1-diphenyl-2-picryl-hydrazyle. Results are presented as the efficacy concentration at 50% (EC_{50}) which is the concentration required to quench half of the DPPH radicals in the solution. The antioxidant vitamin ascorbic acid and flavonoid quercetin are included as references. BQ: betel quid; AN: areca nut; PBI: *P. betle* inflorescence; GB: guava bud; MBR: mangrove bean, raw. Different letters represent significant differences at $p < 0.05$.

of the noni extracts were able to scavenge DPPH, likely due to their low-phenol content. Spearman's coefficient ($r = -0.79$, $p = 0.0062$) indicated a significant association between the total phenol content of an extract and its DPPH-scavenging activity.

In Vitro Inhibition of Cu^{2+}-Mediated LDL Oxidation

To determine whether plant extracts could prolong the lag time before the appearance of conjugated dienes, a product of lipid peroxidation, different concentrations of extract were incubated with LDL and $CuSO_4$ and continuously monitored at 234 nm. In the absence of antioxidants, progressive oxidation and deterioration of the LDL particle occurred approximately 180 min after addition of $CuSO_4$. Lag time was substantially prolonged by extracts with relatively high-phenol content, but only at concentrations higher than 10 μg/mL (Figure 2). Lower concentrations were found to be pro-oxidant. Potent antioxidant activity was demonstrated by BQ because of its PBI constituent, which was able to prolong lag time by 164% at 9 μg/mL relative to LDL oxidized in the absence of extract or standard. The AN component of BQ also displayed an ability to prolong lag time, but only at concentrations higher than 8 μg/mL. The root of noni was the only extract from the plant able to extend lag time, although its effect was inferior in comparison to PBI, AN, BQ, and GB. In contrast, the remaining noni extracts and both mangrove bean extracts were only able to extend lag time for an additional 20 min at concentrations below 5 μg/mL and at higher concentrations, actually enhanced LDL oxidation (data not shown). An exception to this was NJ, which prolonged lag time by 20 min for all concentrations tested (1–25 μg/mL). The same pattern was observed for the positive control, Trolox (data not shown).

Another byproduct of lipid peroxidation of the LDL membrane is the generation of aldehydes, measured as TBARS. Analysis of TBARS formation confirmed that the propagation phase of Cu^{2+}-mediated LDL oxidation occurred after 180 min. As expected, native LDL in the absence of Cu^{2+} did not produce significant TBARS (data not shown). Consistent with our conjugated dienes data, phenolic-rich plant extracts, as well as NR, significantly decreased TBARS levels after 3-h incubation at extract concentrations above 5 μg/mL, except for AN which required 10 μg/mL. Below this concentration, BQ, PBI, AN, and GB exacerbated lipid peroxidation ($p < 0.001$) and increased TBARS levels compared to LDL oxidized in the absence of extracts (Figure 3). The remaining noni extracts, along with both mangrove bean extracts, either promoted TBARS formation or provided no additional protection against LDL oxidation (data not shown). Because these had similar oxidation patterns, the observed activity for MBR was included in Figure 3 as a representative extract.

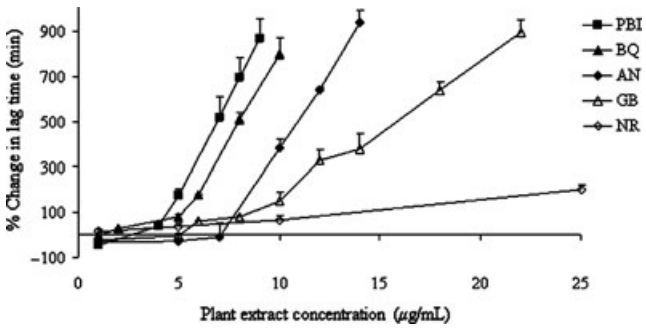

FIGURE 2. In vitro antioxidant activity of various concentrations of selected plant extracts on Cu^{2+}-mediated LDL oxidation measured as lag time (min) before the appearance of conjugated dienes. Results are expressed as percent change in lag time relative to LDL oxidized in the absence of plant extracts (oxLDL). Points above the *x*-axis represent a prolongation of lag time and an increased ability of a plant extract to protect LDL from oxidation. Points below the *x*-axis represent pro-oxidant activity and a shortening of lag time. Results are the average of at least three independent experiments performed in duplicate. BQ: betel quid; AN: areca nut; PBI: *P.betle* inflorescence; GB: guava bud; NR: noni root. All points for plant extract concentrations of 5 μg/mL and higher are significant at $p < 0.05$ vs. oxLDL, except for GB at 5 μg/mL. AN, PBI and GB at 1 μg/mL are significant pro-oxidants at $p < 0.05$ vs. oxLDL.

Effects of Plant Extracts on Cytotoxicity Induced by oxLDL

Loss of cell membrane integrity is an indication of toxicity and ensuing cell death. As a result of increased membrane permeability, the intracellular enzyme LDH is released into the extracellular space and its intra-/extracellular ratio used as a marker of cytotoxicity. Under normal culture conditions, confluent BAEC develop the characteristic "cobblestone" monolayer growth pattern. When oxLDL was added to BAEC, a 13% ($p < 0.0001$) increase in extracellular LDH levels after 6 h relative to BAEC incubated without oxLDL was detected (Figure 4). Histological observations at this stage revealed major morphological cellular alterations. Cellular contraction and elongation were first observed along with cytoplasmic vacuolization, followed by detachment from the bottom of the well. In comparison, native LDL (nLDL) only elicited a 4% increase in LDH leakage, resulting in no observable morphological changes. Our positive control, BHT 10 μM, displayed only a modest effect in reducing oxLDL-induced cytotoxicity.

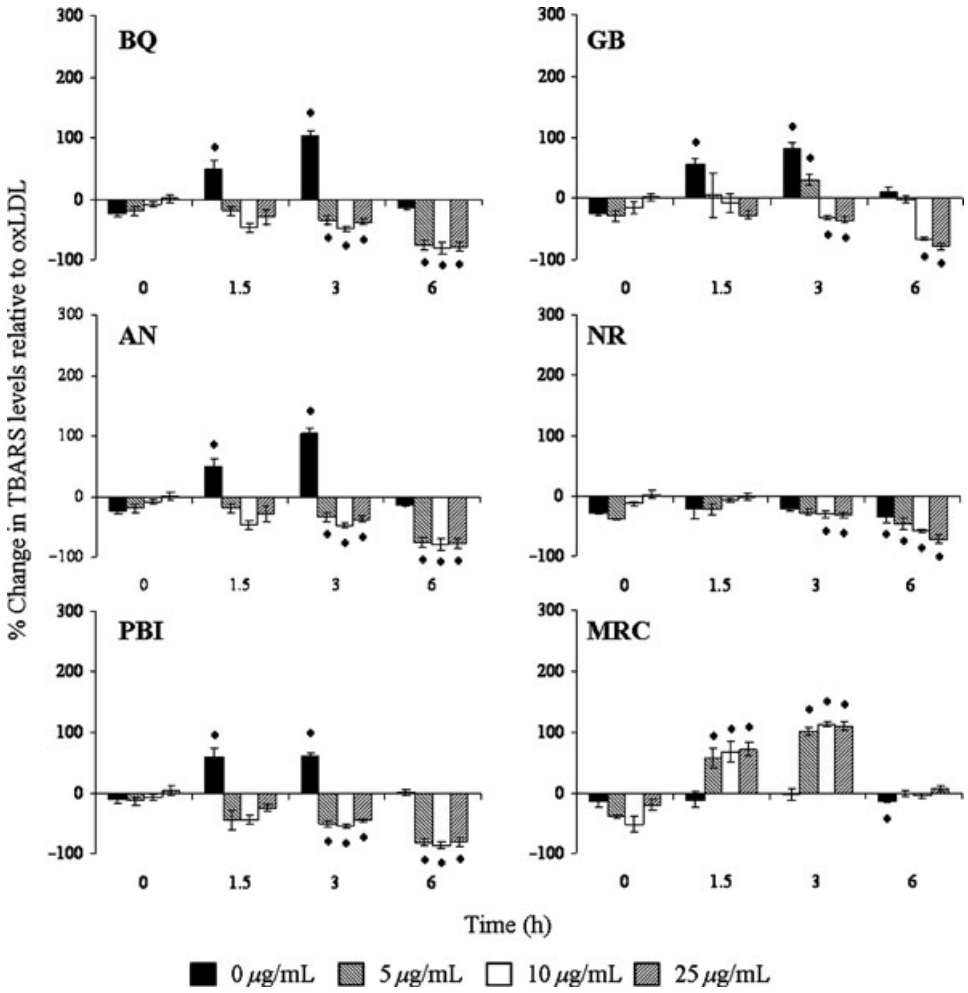

FIGURE 3. In vitro anti- and pro-oxidant activity of various concentrations of selected plant extracts on Cu^{2+}-mediated LDL oxidation measured as TBARS generation over an incubation period of 6 h. Results are expressed as percent change in TBARS generation relative to LDL oxidized in the absence of plant extract (oxLDL). Bars above the *x*-axis represent an increase in TBARS and exacerbation of oxidation compared to normal oxLDL. Bars below the *x*-axis represent antioxidant activity and a reduction in TBARS generation. Results are the average of at least three independent experiments performed in duplicate. BQ: betel quid; AN: areca nut; PBI: *P.betle* inflorescence; GB: guava bud; NR: noni root; MBR: mangrove bean, raw. $^*p < 0.05$ vs. oxLDL.

Of the extracts that had a maximal nontoxic concentration of 10 μg/mL (BQ, AN, PBI, GB and NR), betel quid (BQ) and its ingredients AN and PBI were the only ones that had negligible effects on the cytotoxic action of oxLDL on BAEC, and in the case of PBI, actually increased LDH release after only 3 h ($p = 0.03$). Interestingly, BQ ($7.72 \pm 3.63\%$ increase in LDH) reduced oxLDL cytotoxicity to a degree lower than both its component ingredients AN ($11.64 \pm 2.63\%$) and PBI ($20.12 \pm 4.29\%$), suggesting a possible negating effect resulting from the BQ admixture. The high variance in our data, however, resulted in no statistical difference between BQ and oxLDL after 6-h incubation ($p = 0.109$).

The remaining extracts all displayed the ability to decrease (NF, NL, MBR) or altogether inhibit (GB, NL, NR, MBC) LDH release after 6-h incubation. Our findings establish that processing mangrove bean (MBC) enhances BAEC cytoprotection against oxLDL compared to its raw form (MBR).

Extracellular and Intracellular Production of TBARS in BAEC

Cytotoxicity of cultured BAEC is also characterized by an increase in the lipid peroxidation of the plasma membrane and organelles, resulting in increased generation of extracellular and intracellular aldehydes, respectively, measured as TBARS. Addition of nLDL did not produce significant extracellular or intracellular TBARS for the duration of the incubation time. After

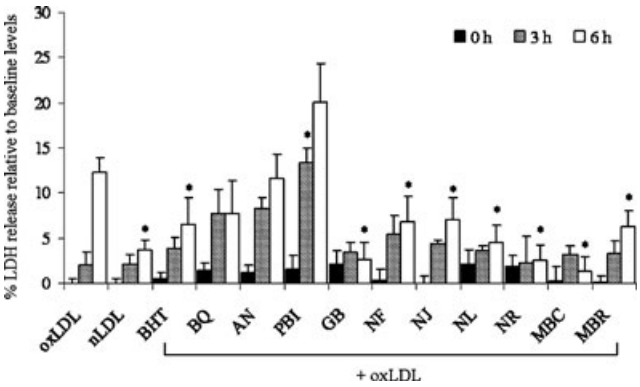

FIGURE 4. Cytotoxicity of maximal nontoxic concentrations of plant extracts in the absence (time 0 h) and presence of 0.10 mg/mL oxLDL in cultured BAEC. Results are presented as percent LDH release relative to normal baseline levels of cells grown in the absence of oxLDL and plant extracts. Bars represent the average ± SEM of at least three independent experiments performed in triplicate. oxLDL: oxidized LDL; nLDL: native LDL; BQ: betel quid; AN: areca nut; PBI: *P.betle* inflorescence; GB: guava bud; NF: noni fruit; NJ: noni juice; NL: noni leaf; NR: noni root; MBC: mangrove bean, cooked; MBR: mangrove bean, raw. The maximal 24 h nontoxic concentration for AN, PBI, BQ, GB and NR was 10 μg/mL; for NF, NJ, NL, MBC, MBR, it was 50 μg/mL. $^*p < 0.05$ vs. oxLDL.

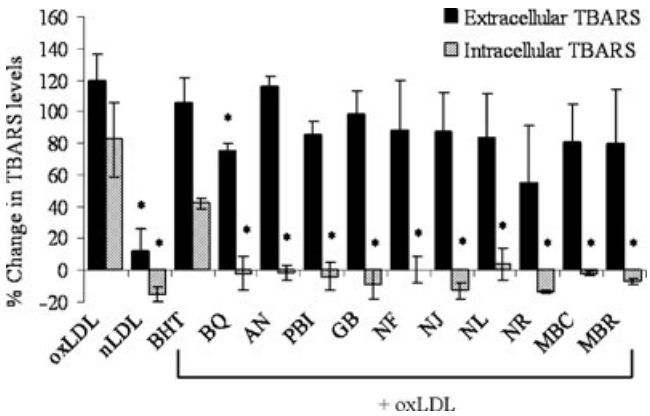

FIGURE 5. Extracellular and intracellular generation of TBARS as a measure of oxidative stress in cultured BAEC incubated for 6 h with 0.10 mg/mL oxLDL and maximal nontoxic concentrations of plant extracts. Results are presented as percent change from baseline TBARS concentrations of cells incubated without oxLDL or plant extracts, and calculated as the mean of at least three independent experiments performed in triplicate. Bars above the *x*-axis represent an increase in TBARS and exacerbation of oxidation compared to normal baseline TBARS levels. Bars below the *x*-axis represent antioxidant activity and a reduction in TBARS. oxLDL: oxidized LDL; nLDL, native LDL; BQ: betel quid; AN: areca nut; PBI: *P.betle* inflorescence; GB: guava bud; NF: noni fruit; NJ: noni juice; NL: noni leaf; NR: noni root; MBC: mangrove bean, cooked; MBR: mangrove bean, raw. The maximal 24 h nontoxic concentration for AN, PBI, BQ, GB and NR was 10 μg/mL; for NF, NJ, NL, MBC, MBR, it was 50 μg/mL. $^*p < 0.05$ vs. oxLDL.

6 h of having added oxLDL to the medium, however, extracellular TBARS levels rose to 120% ($p < 0.001$) and intracellular levels to 83% ($p < 0.01$) relative to BAEC cultured without oxLDL, indicating enhanced oxidative stress. None of the extracts were able to inhibit extracellular TBARS generation except for BQ which had a modest yet statistically significant ($p = 0.03$) protective effect (Figure 5). Every extract tested, however, was able to inhibit intracellular oxidation, indicated by TBARS levels that were lower or no different than BAEC incubated without oxLDL.

DISCUSSION

This study demonstrates the ability of crude plant extracts to protect endothelial cells from oxLDL-induced cytotoxicity is independent of their ability to prevent Cu^{2+}-mediated LDL oxidation. This has potentially important implications when considering the benefits of dietary components vis-à-vis atherosclerosis. The pivotal first step in atherogenesis is considered the oxidative modification of LDL, and the supplementation of exogenous antioxidants has been shown to prevent or delay this process [12]. An important contributor of dietary antioxidants includes polyphenolic phytochemicals that are found abundantly in teas, fruits, and vegetables, the consumption of which is almost always positively associated with reduced risk of chronic disease [13]. When assessed for antioxidant activity in in vitro conditions, however, several dietary phenolic compounds display both antioxidant and prooxidant activity, depending on the structure of the phenol, the functional groups it contains, and its consequential redox potential [14,15]. Extract concentration and the presence of transition metals also determine activity outcome. Likewise, these factors have a heavy influence on cell culture systems, where, depending on the cell line, a compound or extract can exert a cytoprotective or cytotoxic effect [6,16].

On the basis of the DPPH free radical scavenging assay, the Cu^{2+}-oxidized LDL assay and assessment of oxLDL-induced BAEC cytotoxicity, three different patterns of activity could be described: (1) Phenol-rich extracts that inhibited Cu^{2+}-catalyzed LDL oxidation, but were cytotoxic in themselves or exacerbated the cytotoxic properties of oxLDL; (2) Extracts that inhibited Cu^{2+}-catalyzed LDL oxidation and reduced the toxic effects of oxLDL; and (3) Phenol-poor extracts that exacerbated Cu^{2+}-catalyzed LDL oxidation yet were cytoprotective against oxLDL.

Pattern 1: Antioxidant Phenol-Rich Extracts with Cytotoxic Tendencies

Pattern 1 includes the extracts BQ and its two ingredients AN and PBI. These were potent DPPH scavengers and able to inhibit or prolong the onset of Cu^{2+}-mediated LDL oxidation at concentrations above 10 μg/mL but were pro-oxidants at 1 μg/mL (Figures 2 and 3). Both AN and PBI contain phenol hydroxychavicol, a known antioxidant able to effectively scavenge a variety of reactive oxygen species (ROS) [17]. The molecule was also found, however, to induce oxidative stress and cytotoxicity toward cultured Chinese hamster ovary cells and $HepG_2$ cells [18,19]. Similarly, the flavonoids quercetin, eugenol, and isoeugenol, all of which occur in PBI, have been reported to be potent inhibitors of Cu^{2+}-mediated LDL oxidation, but at lower concentrations (10 μM), exacerbated lipid peroxidation [20]. In a previous study, an aqueous extract of PBI exhibited strong ROS-scavenging activity [21] but was also found to promote oxidative stress and cytotoxicity toward cultured oral mucosal fibroblasts and oral keratinocytes, causing DNA breaks [22,23]. The dual anti-/pro-oxidant activity demonstrated by several phenols is due to the compound's redox potential at a given concentration that favors the formation of the stable phenoxyl radical, which is able to further react with lipid peroxides [14].

Transition metals are critical to the in vitro oxidation of LDL, but their role under physiologically plausible conditions is not clear [10]. Since Cu^{2+} was added simultaneously with the extracts in our in vitro assays, it is possible that some plant phenols were able to chelate Cu^{2+}, thus acting as indirect antioxidants by inhibiting binding of the metal to LDL. This is particularly relevant for tannins, phenolic macromolecules able to bind and sequester a number of compounds, including many free radicals and transitional metals [24]. Tannins are especially abundant in AN and may account for its antioxidant activity. However, AN also contains a relatively high concentration of the transitional metals copper (3–188 μg/g dry weight) and iron (~75 μg/g) [25] which can exacerbate the oxidation of phenols and lipids. This would explain why a higher concentration of AN (8 μg/mL), relative to the other phenol-rich extracts, was needed to convert the extract from a pro-oxidant to an antioxidant in our LDL oxidation assays (Figures 2 and 3). The AN phenol/transition metal ratio at concentrations above 8 μg/mL shifted the extract's redox potential toward antioxidation.

In our cell culture experiment conditions, cells were preincubated for 24 h with the extract, the medium removed and the extract reintroduced along with extensively oxidized LDL. It is important to note that Cu^{2+} added singly to BAEC did not induce significant LDH release or TBARS generation after 6-h incubation (data not shown). Therefore, the source of toxic compounds came from the oxLDL particle (such as oxidized polyunsaturated fatty acids (PUFA), oxysterols, and aldehydes), extract phytochemicals or the product of their biochemical interaction. In the case of AN, PBI and BQ, any concentration above 10 μg/mL was found to be cytotoxic after 24-h incubation with BAEC. At this maximal nontoxic concentration, none were able to offer any additional benefit in protecting BAEC from oxLDL-induced cytotoxicity, and in the case of PBI, exacerbated the condition after only 3 h (Figure 4).

Interestingly, the in vitro pro-oxidative tendencies of AN and PBI at 1 μg/mL were substantially reduced when combined to form BQ. No effect on lag time was observed for BQ at 1 μg/mL (Figure 2) and TBARS production after 6h was lower than either AN or PBI (Figure 3), suggesting a sort of synergistic action. This effect was also observed in our cell culture experiment where BQ was less cytotoxic than AN and PBI and was the only extract able to reduce extracellular TBARS formation 6 h after addition of oxLDL. Given the content of metal ions in AN and the polyphenol content in PBI, the resulting redox potential of BQ essentially depended on the balance between the rate of free radical generation from AN metals and the rate at which they could be quenched by AN and PBI polyphenols. The presence of slaked lime potentially affected the redox potential of phenols by raising the pH. In alkaline conditions (pH ≥ 9.5), areca polyphenols rapidly undergo autoxidation to form $O_2^{\bullet-}$ and H_2O_2, a reaction that is aggravated by the presence of metal ions [26]. Our data demonstrated that BQ had an antioxidant capacity between that of PBI and AN, but much closer to that of the former, indicating that PBI polyphenols could easily contend with the enhanced presence of metal ions.

Pattern 2: Antioxidant Extracts with Cytoprotective Effects

Pattern 2 includes the phenol-rich extract GB and the phenol-poor root extract of the noni tree, NR. Both significantly prolonged the antioxidative capacity of LDL against Cu^{2+}oxidation (Figures 2 and 3) and inhibited oxLDL cytotoxicity toward BAEC (Figure 4). GB shares a common property with AN and PBI, in that it displayed pro-oxidant activity at low concentrations (1 μg/mL), indicated by a decrease in lag time (Figure 2) and a rise in TBARS (Figure 3) from Cu^{2+} oxidation of LDL. One of the more abundant phenolic compounds in GB is quercetin [27], which would explain the pro-oxidant/antioxidant duality of the extract, as well as its potent DPPH-scavenging

capacity. In a previous report, an extract of guava leaf was found to possess equipotent free radical scavenging activity to quercetin [28]. Unlike AN and PBI, however, GB exhibited a cytoprotective tendency and inhibited oxLDL cytotoxicity toward BAEC after 6 h (Figure 4), a property that is likely due to its content of the phenol (+)-gallocatechin. This compound was previously identified as a strong antimutagen, able to protect *Escherichia coli* from UV-induced mutations [29]. This was attributed to the molecular structure of (+)-gallocatechin, which contains three adjacent hydroxyl groups; an apparent requirement for phenols to possess this activity. It is plausible that (+)-gallocatechin could equally have protected BAEC from oxLDL-induced cytotoxicity, although further studies are necessary to confirm this.

Noni root extract stands apart from the remaining noni extracts obtained from the leaf (NL), fruit (NF), and fruit juice (NJ) in that it contains a greater content of anthraquinones, a group of compounds that possess antioxidant or pro-oxidant activity depending on the number and position of hydroxyl groups and glycosides on the phenolic structure [30]. Our results mirror that of Zin et al. [31,32] who observed antioxidant activity in the MeOH extract of NR, but not NF or NL using the ferric thiocyanate method (FYC) and TBARS assay. In their study, further fractionation and retesting of the extracts found no correlation between phenol content and antioxidant activity, suggesting the presence of nonphenolic antioxidants. Although clearly an antioxidant according to our LDL oxidation studies, NR was inferior compared to the phenol-rich extracts AN, PBI, BQ and GB, and possessed virtually no DPPH-scavenging ability. In an earlier study, a lack of free radical scavenging activity was also observed for the anthraquinone-rich roots of *Rumex patienta* [33]. Anthraquinones have been reported to be cytotoxic or cytoprotective, depending on such familiar factors as concentration, chemical structure and the cell line used. At relatively high concentrations, anthraquinones are thought to induce oxidative stress in hepatocytes, which might account for the few case reports of hepatoxicity in those who overly consumed noni [34]. On the other hand, when an anthraquinone-rich plant extract was incubated in isolated perfused rat hearts, a dose-dependent protective effect against ischemia-reperfusion injury was observed as evidenced by a significant decrease in LDH leakage. Protection in this case was found to be associated with enhanced myocardial glutathione status [35]. In the present study, NR extract concentrations greater than 10 μg/mL was found to be cytotoxic toward BAEC, but at lower concentrations was able to protect the cells from oxLDL-induced injury. In light of what is known about the biological effects of anthraquinones, they could account for the properties exhibited by NR in this study.

Pattern 3: Pro-Oxidant Extracts with Cytoprotective Effects

Pattern 3 includes the remaining noni extracts NF, NJ and NL, as well as the raw (MBR) and cooked (MBC) extracts of the mangrove bean. These were relatively poor in phenol content (Table 1) and lacked the ability to scavenge the DPPH radical (with the exception of tannin-rich MBR, which had an EC_{50} similar to quercetin and the other phenol-rich extracts AN, PBI, BQ and GB). None were able to protect LDL from Cu^{2+} oxidation (Figures 2 and 3), and with the exception of NJ, actually shortened lag time and exacerbated TBARS generation with increasing extract concentration. Yet at a concentration of 50 μg/mL, all were able to inhibit oxLDL-induced cytotoxicity toward BAEC for up to 6 h (Figure 5).

In an earlier study, a 50 μg/mL MeOH and EtOAc fraction of noni juice was reportedly able to inhibit 88 and 96%, respectively, in vitro TBARS production from Cu^{2+}-catalyzed LDL oxidation. The isolated active compounds were found to be a series of lignans whose potency depended on the number of phenolic hydroxyl groups [36,37]. Similarly, a lignan isolated from the *n*-BuOH soluble portion of noni fruit was found to be a potent DPPH and $ONOO^-$ scavenger [38]. These findings are contradictory to ours, in which noni juice demonstrated very poor DPPH-scavenging activity. This discrepancy may be due to differences in sample preparation since we used the juice in its commercial form rather than as an extraction, which may have diluted any antioxidant lignans. The juice did have a modest antioxidant effect in prolonging lag time (16% longer at 25 μg/mL) before the appearance of conjugated dienes. However, TBARS production suggested a pro-oxidative effect with increasing concentration (data not shown). Likewise our NF extract, which may more closely relate to the juice extract used by Kamiya et al. [36], also displayed poor DPPH-scavenging activity. A lack of antioxidant activity in a MeOH extract of NF was also reported by Zin et al. [32]. In the present study, antioxidant effects were noted at low concentrations (<5 μg/mL), where 1 μg/mL prolonged lag time by 26% and reduced TBARS by 20%, but at higher concentrations displayed pro-oxidant activity, shortening lag time by 29% at 25 μg/mL, a concentration half of that used by Kamiya et al.

Our results for NL extract concur with the findings of Salleh et al. [37], who reported no LDL protective effects against Cu^{2+} catalysis and only 5% TBARS inhibition at a concentration of 12.5 μg/mL. Similarly, a

NL MeOH extract failed to exhibit antioxidant activity using the ferric thiocyanate method (FYC) and TBARS assay, although like NF, the ethyl acetate fraction was more active [32]. In this study, a similar NL concentration as that used by Salleh et al. (10 μg/mL) shortened lag time by a modest 14% ($p < 0.01$), but had no effect on reducing TBARS after 6 h. Like the roots (NR), noni fruit (NF), leaves (NL), and juice (NJ) contain antioxidant anthraquinones, albeit in much smaller quantities, which is why their maximal nontoxic concentration was 50 μg/mL rather than the 10 μg/mL required for NR. Differences in anthraquinone concentration may account for the observed pro-oxidant activity in our LDL oxidation experiments, but this did not affect the extracts' ability to inhibit oxLDL-induced cytotoxicity. All were able to prevent LDH leakage after 6-h incubation with oxLDL.

In regard to mangrove bean, our study demonstrates that cooking of the bean via traditional methods removes approximately 80% of the total phenol content occurring in the raw plant, causing a complete loss of DPPH-scavenging activity, but without otherwise affecting its activity toward LDL oxidation and BAEC cytoprotection. Nutritional composition analysis of mangrove propagule shows that it contains 20–50 g starch/100 g dry weight [39]. Considering that mangrove starches are more highly branched and viscous compared to other common food sources, it is possible that it prevented the binding of oxLDL to BAEC by either sequestering oxLDL or creating a mechanical barrier via cross-linking with the endothelial surface glycocalyx. Tannins, likewise, would have this same ability and may have been the responsible agent for the cytoprotection displayed by MBR. In the same way, mangrove polysaccharides and tannins could bind to endothelial surfaces, causing alterations in membrane permeability and permselectivity, thus preventing LDH leakage into the culture medium.

Internalization of oxLDL is mediated by the lectin-like LDL receptor-1 (LOX-1) and is immediately associated with an increase in intracellular oxidative stress and TBARS [4]. Intense and sustained rises in cytoplasmic Ca^{2+} and mitochondrial generation of ROS [40] following oxLDL uptake trigger the activation of degradative enzymes resulting in irreversible damage of cellular components and eventual cell necrosis [41]. In the present study, all extracts were able to block intracellular signaling pathways leading to oxidative stress. In light of the findings of Vieira et al. [6], who noted that certain phenols were unable to integrate into endothelial cells, it is plausible that the extracts blocked an early event in the signaling pathway, perhaps at the ligand-binding site. Although all extracts were able to prevent an intracellular rise in TBARS, none were able to contend with the pro-oxidant effects of oxLDL on BAEC lipid membranes after 6-h incubation, represented as extracellular TBARS (Figure 5). We know that TBARS in the culture medium did not arise from intracellular sources and subsequently leaked into the medium since there was no correlation between LDH leakage and extracellular TBARS 6-h postincubation. Also, TBARS could not have come from the oxLDL particle or they would have been detected in earlier (3 h) measurements (data not shown). The lack of association between antioxidant activity and cytoprotection in this study is supported by the lack of activity seen with our positive antioxidant control, BHT. This, however, may be a question of dosage, since a previous study using fibroblasts found that 10 μM BHT had no effect on inhibiting oxLDL-induced toxicity [42], whereas a higher dose (50 μM) proved effective [43].

In light of these findings, the association between antioxidant-rich plants and health may not be as strong as purported. Indeed, some antioxidant compounds such as those in AN and PBI may inhibit peroxidation, but the resulting phenoxyl radicals can exert cytotoxic or mutagenic effects. Likewise, some plant extracts such as NF, NJ, NL, and MBC may be able to protect endothelial tissues despite being poor radical scavengers. Clearly, antiatherogenic factors need not be antioxidant in nature as long as vascular tissues can be protected from the cytotoxic presence of oxLDL.

CONCLUSIONS

In the present study, a preliminary examination of the effects of various plant extracts on LDL oxidation and cytoprotection of cultured BAEC in the absence and presence of oxLDL was undertaken without further elucidation of biomechanisms. Methanolic extracts of areca nut (AN), *P. betle* inflorescence (PBI) and betel quid contained high concentrations of antioxidant polyphenols able to protect LDL from Ca^{2+}-catalyzed oxidation, but had no effect on oxLDL-induced endotheliopathy. Guava bud and noni root extract were not only able to effectively inhibit LDL oxidation in a dose-dependent manner, but also prevented oxLDL cytotoxicity for up to 6 h. With the exception of MBR, the remaining extracts NF, NJ, NL, and MBC were poor DPPH scavengers because of their low-phenol content. All were unable to protect LDL from oxidation, except NJ which prolonged antioxidant protection for an additional 20 min. All noni and mangrove bean extracts, however, were able to protect BAEC from oxLDL cytotoxicity. From these observations, we can conclude that the antioxidant ability of a plant extract is independent of its ability to protect BAEC from the toxic byproducts of extensively oxidized LDL. Further research into functional dietary compounds able to mediate

atherogenesis is required to develop culturally specific strategies to maintain cardiovascular health.

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