Free Rad. Res., Vol. 23, No. 2, pp. 175–184
Reprints available directly from the publisher
Photocopying permitted by license only

MANGOSTIN INHIBITS THE OXIDATIVE MODIFICATION OF HUMAN LOW DENSITY LIPOPROTEIN

PETA WILLIAMS,[1] METTA ONGSAKUL,[2] JULIE PROUDFOOT,[1] KEVIN CROFT[1] and LAWRIE BEILIN[1]

[1]*University of Western Australia, Department of Medicine at Royal Perth Hospital, Australia* [2]*Faculty of Science, Prince of Songkla University, Hatyai 90112, Thailand*

(Received September 13th, 1994; in revised form, October 21st, 1994)

The oxidation of low density lipoprotein (LDL) may play an important role in atherosclerosis. We investigated the possible antioxidant effects of mangostin, isolated from *Garcinia mangostana*, on metal ion dependent (Cu^{2+}) and independent (aqueous peroxyl radicals) oxidation of human LDL. Mangostin prolonged the lagtime to both metal ion dependent and independent oxidation of LDL in a dose dependent manner over 5 to 50 μM as monitored by the formation of conjugated dienes at 234nm ($P < 0.001$). There was no significant effect of mangostin on the rate at which conjugated dienes were formed in the uninhibited phase of oxidation. Levels of thiobarbituric reactive substances (TBARS) generated in LDL were measured 4 and 24 hours after oxidation with 5μM Cu^{2+} in the presence or absence of 50μM or 100μM mangostin. We observed an inhibition of TBARS formation with 100μM mangostin at 4 hours ($P = 0.027$) but not at 24 hours ($P = 0.163$). Similar results were observed in the presence of 50μM mangostin. Mangostin, at 100μM, retarded the relative electrophoretic mobility of LDL at both 4 and 24 hours after Cu^{2+} induced oxidation. Mangostin (100μM) significantly inhibited the consumption of α-tocopherol in the LDL during Cu^{2+} initiated oxidation over a 75 minute period ($P < 0.001$). From these results, we conclude that mangostin is acting as a free radical scavenger to protect the LDL from oxidative damage in this *in vitro* system.

KEY WORDS: mangostin, low density lipoprotein, oxidation

INTRODUCTION

Growing evidence suggests a role for the oxidative modification of low density lipoprotein (LDL) in atherogenesis.[1,2] Oxidation causes multiple changes in both the lipid and protein constituents of LDL facilitating recognition by the scavenger receptors of macrophages and potentially leading to lipid accumulation and foam cell formation.[3] Oxidised LDL also exhibits other potentially atherogenic properties, for example, it is chemotactic for monocytes, stimulates leukocyte adhesion to vascular endothelium and is cytotoxic.[1,2]

Animal experiments have reported that lipophilic antioxidants such as probucol and butylated hydroxytoluene can inhibit the development of atherosclerotic lesions in cholesterol fed rabbits[4,5] and inhibit LDL oxidation *in vitro*.[6] Alpha-tocopherol[7] and flavonoids such as (+)-catechin[8] have also been shown to inhibit the oxidation of LDL. These phenolic antioxidants interfere with lipid peroxidation by donating a hydrogen atom to effectively terminate the lipid radical with the consequent formation of a

Address for Correspondence: Peta Williams University Department of Medicine Royal Perth Hospital Box X2213 GPO, Perth, 6001, WA Australia telephone: (09) 224 0245 fax: 61 9 224 0246

relatively inactive resonance stabilised phenoxy radical.[9] Recently, it has been suggested that inhibition of LDL oxidation by phenolic substances in red wine may help explain the 'French paradox' of high fat diets with low incidence of coronary atherosclerosis.[10] The potential of naturally occurring compounds, particularly those occurring in the diet, that may inhibit LDL oxidation is an area of considerable interest. Mangostin (Figure 1), is a constituent of the mangosteen fruit of the tropical tree *Garcinia mangostana*, a popular and widely consumed fruit in South East Asia. This compound has been previously reported to have some antimicrobial activity.[11] The structure of mangostin suggests that it may act as a free radical scavenger and thereby, potentially inhibit the oxidative modification of the lipoproteins. In this study we have investigated the effect of mangostin on metal ion dependent and independent *in vitro* peroxidation of LDL.

MATERIALS AND METHODS

Mangostin was extracted from the dried skin of the mangosteen fruit of *Garcinia mangostana* and purified by recrystallisation (melting point 180–182°C, literature 181.6–182.6°C).[12] The structure of mangostin is shown in Figure 1.

LDL was isolated from EDTA plasma by density gradient ultracentrifugation.[13] Samples were ultracentrifuged at 296 000xg (average) for 4 hours using a Centrikon T-1190 Ultracentrifuge (Kontron Instruments, Milano, Italy). The LDL band (density 1.006–1.063g/ml) was collected by aspiration and passed through a Pharmacia PD10 Sephadex column to remove the excess salt and the majority of the EDTA. The LDL was stored at 4°C in the dark and used within 1 week. Isolated LDL was passed through a Pharmacia PD10 Sephadex column to remove any remaining EDTA just prior to the oxidation experiments. Mangostin in ethanol was added immediately prior to the initiation of oxidation and the total addition of ethanol was never more than 1% (v/v). This concentration of ethanol was added to the controls.

We examined the production of conjugated dienes over time using the method described by Esterbauer *et al.*[14] which has been used by us previously.[15] The cholesterol content of the LDL was measured using a standard enzymatic method (Monotest, Boehringer Mannheim, Germany) and the LDL diluted with 0.15M phosphate buffered saline (PBS) to a standard concentration of 0.3 mmol/L cholesterol. Oxidation was initiated by the addition of $CuCl_2$ (final concentration 5μM) or 2,2′-azobis-(2-amidinopropane)hydrochloride (AAPH, final concentration 4mM). AAPH is a water soluble compound that thermally decomposes to generate peroxyl radicals at a constant and known rate.[16] Oxidation kinetics were determined by monitoring the change

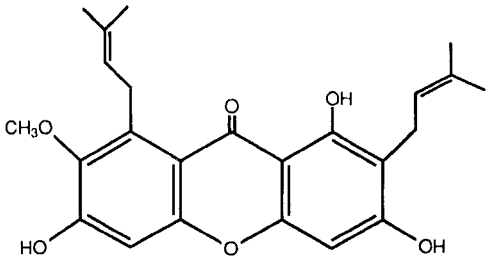

FIGURE 1 The structure of mangostin.

in absorbance at 234nm using a DU650 UV-Vis spectrophotometer (Beckman Instruments Inc, CA, USA) with absorbance readings every 20 minutes over 240 minutes at a controlled temperature of 30°C for the Cu^{2+} and 37°C for the AAPH experiments. The plot of absorbance against time was divided into a lag phase and a propagation phase. The lag time was defined as the intercept between the tangent of the absorbance curve during the propogation phase with the baseline and was expressed in minutes. The rate of oxidation was calculated from the slope of the absorbance curve during the propagation phase and was expressed as Δ absorbance units/minute.

In separate experiments, LDL was diluted 1:1 with PBS and oxidation initiated by the addition of $CuCl_2$ (final concentration 5μM). At 0, 1, 2, 4 and 24 hours after initiation of oxidation, LDL was sampled and aliquots added to EDTA (final concentration of 1mg/ml) to terminate the reaction. Thiobarbituric acid reactive substances (TBARS) and relative electrophoretic mobility were assessed on these samples. For the TBARS assay, an equal volume of 25% (w/v) trichloroacetic acid was added to each sample followed by an equal volume of 1% (w/v) thiobarbituric acid. The mixture was heated for 30 minutes in a boiling water bath. After cooling on ice, the samples were centrifuged in a microfuge at 10 000rpm for 10 minutes and the absorbance of the clear supernatant measured at 532nm on a DU 650 Spectrophotometer (Beckman Instruments Inc, CA, USA). The amount of TBARS was determined by comparison to a standard curve using malondialdehyde generated by serial dilution of 1,1,3,3-tetramethoxypropane (Sigma-Aldrich, Australia) in 0.05M H_2SO_4 immediately prior to assay. These results were expressed in nmol equivalents of malondialdehyde per mg of LDL protein. LDL protein levels were measured by the method of Lowry[17] using bovine serum albumin as the standard.

Relative electrophoretic mobility of native and oxidised LDL was assessed using the Paragon Electrophoresis System for Lipoprotein electrophoresis (Beckman Instruments Inc, Fullerton, CA, USA). Migration was initially expressed relative to native LDL and is presented as a percentage of control.

To measure consumption of α-tocopherol levels with oxidation, we diluted LDL 1:1 with PBS and initiated oxidation with 2μM $CuCl_2$ at room temperature. Samples were taken at 15 minute intervals for 75 minutes. Alpha-tocopherol levels in LDL were measured by reverse phase HPLC using electrochemical detection using a Bioanalytical Services LC-4B amperometric detector with an applied potential of +0.5V and a range of 50na. An equal volume of tocopherol acetate (37.5μM) in ethanol was added to the LDL sample as an internal standard and the samples immediately extracted into hexane. Samples were then dried down under N_2 and reconstituted in the mobile phase (50% methanol, 50% ethanol, 0.2M NaClO). A 25cm C18 Nucleosil column (Alltech) and a flow rate of 1ml/min was used. Results are expressed in μM.

Statistical analysis was carried out with the Statistical Package for the Social Sciences V5 (SPSS Inc, Chicago, USA) with results presented as means ± sem. One way analysis of variance was used to compare the effects of various doses of mangostin on lagtime and rate. Paired t-tests were used to compare the effects of mangostin and vehicle on TBARS production. Regression analysis was used to compare between the alpha-tocopherol decay curves with mangostin or with vehicle.

RESULTS

Figures 2 and 3 are representative of time courses for the formation of conjugated dienes in LDL after initiation of peroxidation with 5μM Cu^{2+} or 4mM AAPH,

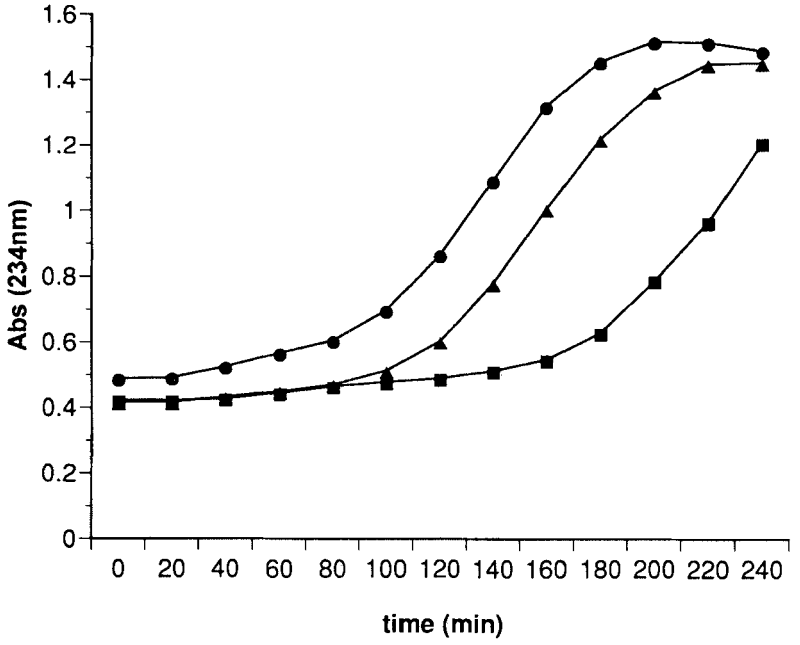

FIGURE 2 Representative trace demonstrating the change in absorbance (234nm) over time (minutes) in LDL after initiation of peroxidation with Cu^{2+} (30°C) in the presence or absence of mangostin. LDL plus vehicle (closed circles), LDL plus 20μM mangostin (closed diamonds), LDL plus 50μM mangostin (closed squares).

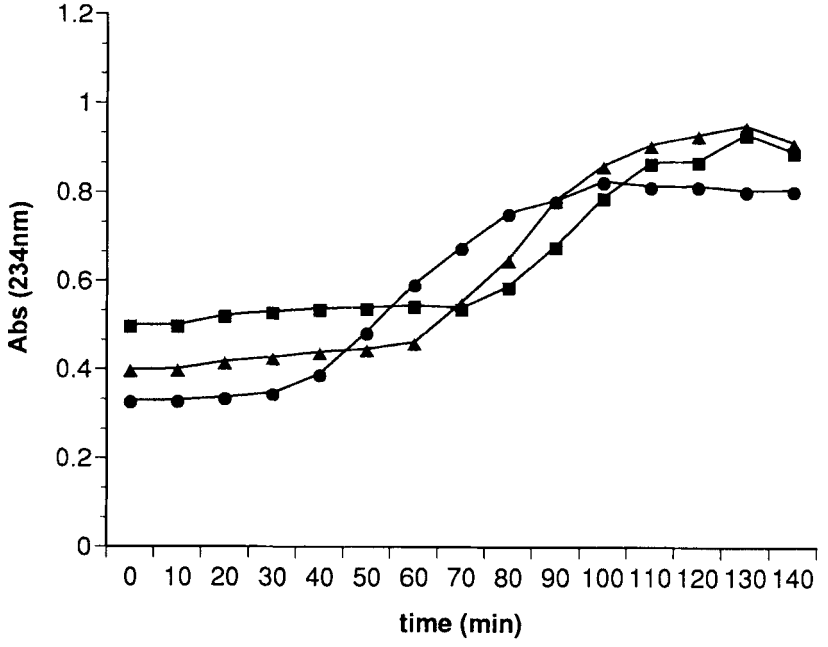

FIGURE 3 Representative trace demonstrating the change of absorbance (234nm) over time (minutes) in LDL after initiation of oxidation with AAPH (37°C) in the presence or absence of mangostin. LDL plus vehicle (closed circles), LDL plus 20μM mangostin (closed diamonds), LDL plus 50μM mangostin (closed squares).

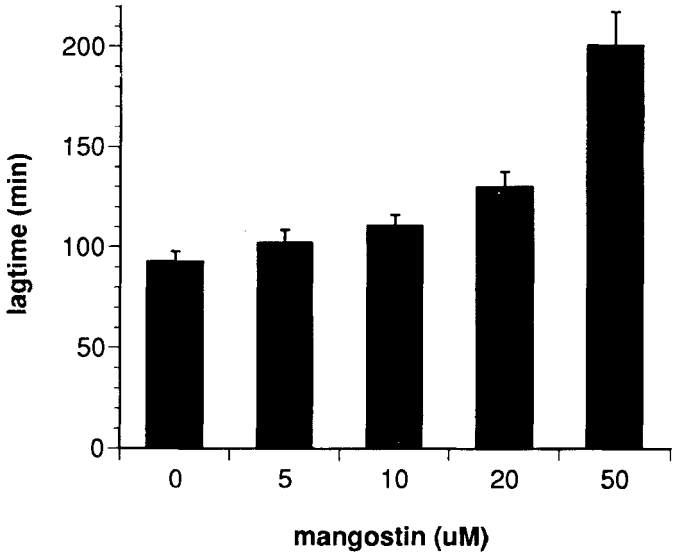

FIGURE 4 The effect of increasing dose of mangostin (μM) on lagtime (minutes) to Cu^{2+} initiated LDL oxidation. Statistical analysis was by oneway analysis of variance ($F_{4,20} = 21.51$, $P < 0.001$, $n = 5$).

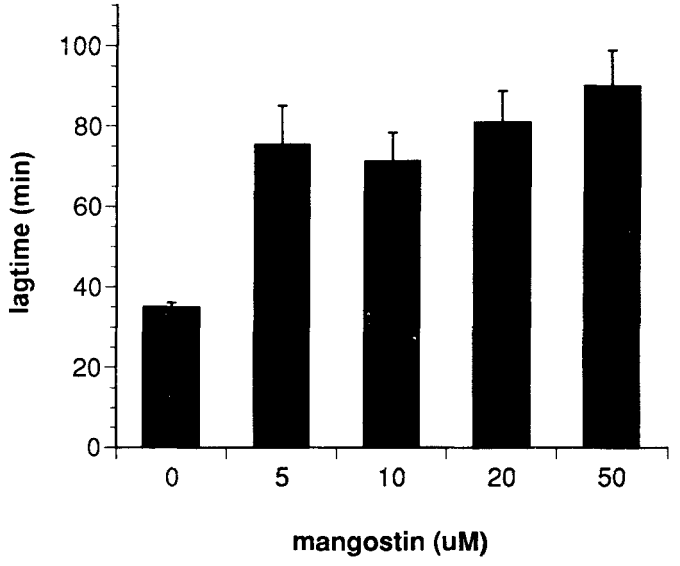

FIGURE 5 The effect of increasing doses of mangostin (μM) on lagtime (minutes) to AAPH initiated LDL oxidation. Statistical analysis was by oneway analysis of variance ($F_{4,25} = 7.688$, $P < 0.001$, $n = 6$)

respectively. Mangostin prolonged lagtime to Cu^{2+} (Figures 2, 4) and AAPH (Figures 3, 5) induced oxidation of LDL in a dose dependent manner. There was no significant effect of mangostin on the rate of formation of conjugated dienes with Cu^{2+} (Figure 2, $F_{4,18} = 1.71$, $P = 0.191$, $n = 5$) or AAPH initiation (Figure 3, $F_{4,25} = 0.008$, $P = 1.00$, $n = 6$). We did not go higher than the 50μM dose with the conjugated diene method as mangostin absorbs in the ultraviolet region and high doses led to a considerable signal to noise ratio. To further investigate the potential antioxidant effects of this compound, TBARS formation was measured in LDL oxidised with Cu^{2+} in the presence of 50μM or 100μM mangostin. Both doses of mangostin protected LDL from oxidation at 4 hours but not 24 hours (Table 1). The TBARS levels at 0, 1 and 2 hours were below the limits of sensitivity in both the control and mangostin treated LDL (data not shown).

Peroxidation of LDL results in changes in electrophoretic mobility due to modification of the lysine residues on the apoprotein B moiety and consequent alterations in surface charge.[3] The relative electrophoretic mobility of LDL oxidised in the presence of 100μM mangostin was approximately 53% and 72% of the migration observed in the absence of mangostin at 4 and 24 hours, respectively (Figure 6). Inhibition of mobility was also observed with the 50μM dose of mangostin compared to control (60% and 80% at 4 and 24 hours, respectively). There was no observable difference from baseline at 1 or 2 hours for either the treated or control LDL (data not shown).

Alpha-tocopherol is the major lipid soluble antioxidant present in LDL.[7] As the decay of alpha-tocopherol levels in LDL supposedly occurs prior to significant oxidation, we examined the effect of mangostin on the consumption of α-tocopherol upon Cu^{2+} dependent LDL oxidation. A dose of 100μM mangostin significantly inhibited the consumption of α-tocopherol compared to controls ($P < 0.001$, Figure 7). Mangostin at 50μM tended to inhibit the decay of α-tocopherol but did not have a statistically significant effect (data not shown).

DISCUSSION

The results from the present study demonstrate that mangostin can effectively protect LDL against Cu^{2+} induced lipid peroxidation as evidenced by the increase in lag time with increasing dose of mangostin and the relative decrease in TBARS in the mangostin treated LDL at 4 hours compared to control. In addition, mangostin inhibited AAPH derived peroxyl radical mediated, i.e. metal ion independent, LDL oxidation. These results, together with the phenolic structure of mangostin (Figure 1), are consistent with mangostin acting as a conventional free radical scavenger in this *in vitro* system

TABLE 1.
The level of TBARS generated in LDL at 4 and 24 hours after oxidation with 5μM copper at 37°C in the presence or absence of mangostin.

	TBARS (nmol MDA eq/mg LDL-protein)			
	MANGOSTIN		CONTROL	
Time (hrs)	4	24	4	24
50μM	25.3 (7.4)	25.0 (8.3)	32.3 (7.8)**	22.3 (7.7)
100μM	34.8 (5.7)	32.3 (8.1)	46.9 (8.6)***	27.3 (5.5)*

Results are mean (sem), n = 4 for each group at each dose.
Statistical analysis was by paired t-test comparing mangostin with control at both 4 and 24 hours, respectively, *P = 0.048, **P = 0.027, ***P = 0.018.

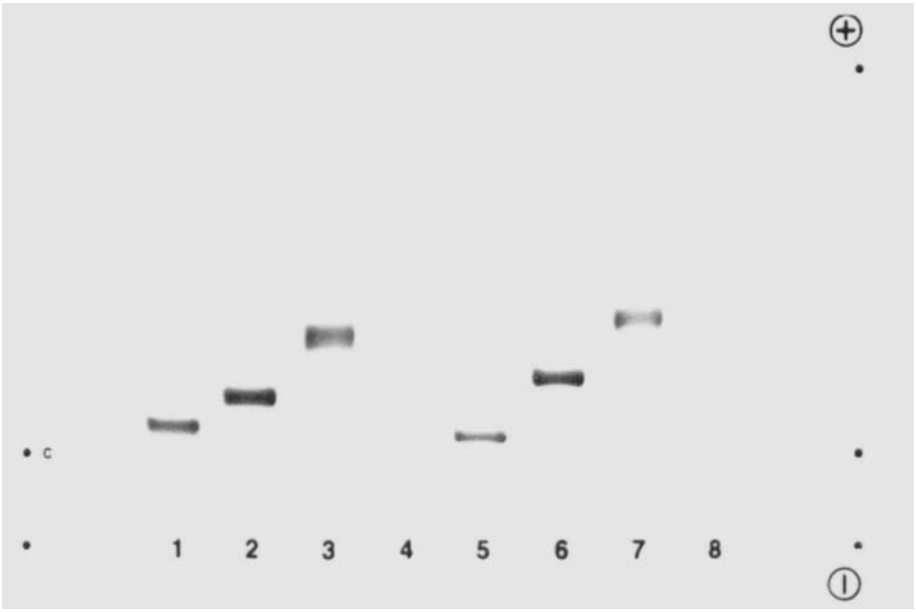

FIGURE 6 Representative gel demonstrating the effect of 100μM mangostin on relative electrophoretic mobility of LDL after oxidation with 5μM $CuCl_2$.

Lane 1, 2 and 3: LDL in PBS with 5μM $CuCl_2$ and 100μM mangostin at 0 time, 4 hours and 24 hours, respectively.

Lane 5, 6 and 7: LDL in PBS with 5μM $CuCl_2$ and vehicle at 0 time, 4 hours and 24 hours, respectively.

protecting the lipoprotein from oxidative damage. The observation that the presence of mangostin spares the consumption of α-tocopherol in the LDL in the early oxidation period would suggest that mangostin is either adding more total scavenging potential to the LDL or trapping the free radicals in preference to the endogenous α-tocopherol. The lack of effect of mangostin on the rate of formation of conjugated dienes during the uninhibited phase of oxidation suggests that once the available scavenging potential of the mangostin and other endogenous anti-oxidants is consumed, the reaction proceeds to completion unhindered.

We observed an effect of mangostin on TBARS formation at 4 hours but not 24 hours after the initiation of the reaction. Since malondialdehyde formation is a relatively late step in lipid peroxidation, this supports the suggestion that mangostin adds to the free radical scavenging potential and extends the initial resistance to oxidation until the antioxidants are exhausted. Similarly, Jialal *et al.*[18] found that the oxidation of LDL with Cu^{2+} was maximal by 4 hours (by TBARS) and that α-tocopherol protected LDL at 5 but not 24 hours. The TBARS level at 24 hours was lower than at 4 hours probably due to the polyunsaturated substrate being consumed and the decomposition of the malondialdehyde end product.

Oxidation of LDL results in changes in electrophoretic mobility due to modification of the ε-amino lysine residues on the apoprotein B moiety and consequent alterations in surface charge.[3] Mangostin inhibited the relative electrophoretic mobility of oxidised LDL at both 4 and 24 hours compared to control but did not completely hinder the formation of modified lysine residues. This indicates that while 100μM mangostin

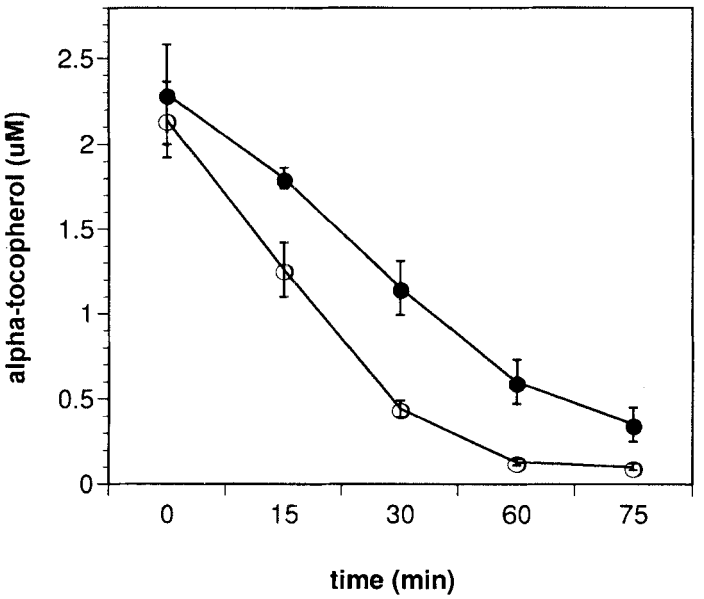

FIGURE 7 Effect of 100μM mangostin on the consumption of α-tocopherol in LDL after initiation of oxidation with 2μM $CuCl_2$. Closed circles represent LDL with 100μM mangostin and open circles represent LDL with vehicle. Statistical analysis was by regression ($F_{3,26} = 90.42$, $P < 0.001$, $n = 3$).

confers some protection to LDL, there is still modification of the apoprotein. Mangostin inhibited the relative mobility considerably more at 4 than 24 hours further supporting the hypothesis that mangostin acts early to extend the resistance to oxidation.

Naturally occurring phenolic compounds, such as the flavonoids, have been shown to bind Cu^{2+} ions.[19] Thus, mangostin may exert part of its action by binding the initiating Cu^{2+} ions and thereby reducing the formation of free radicals. However, our finding that mangostin dose dependently increases lagtime when oxidation is initiated by the thermal decomposition of AAPH, a metal ion independent mechanism, suggests that this compound is scavenging peroxyl radicals and implies that chelation of Cu^{2+} does not explain all the antioxidant effect of mangostin.

Recently, 50μg/ml (+)-catechin (approx. 175μM), a naturally occurring flavonoid, was shown to inhibit Cu^{2+} induced oxidation of LDL, as indicated by TBARS, to 20% of control.[8] However, considerable differences in methodologies preclude any direct comparison between the efficacy of mangostin and (+)-catechin. In the present study, mangostin showed a varying degree of inhibition to oxidation which depended on the particular methodology. Oxidation initiated with AAPH was inhibited by 5μM mangostin while 10–20μM significantly prolonged lagtime to oxidation with Cu^{2+} and 100μM mangostin inhibited TBARS production by about 20%. The literature suggests that no single assessment of lipid peroxidation is best and that a number of methods should be employed when evaluating this complicated phenomenon. Probucol, another phenolic antioxidant, has been demonstrated to inhibit the oxidative modification of LDL by endothelial cells to a greater extent than it does when LDL is oxidised by 5μM Cu^{2+}.[6]

While the majority of the mangostin is found in the skin of the mangosteen, it is also present in the flesh of the fruit [Ongsakul, personal communication]. Details on the consumption and absorption of mangostin from the fruit and its metabolism *in vivo* are currently unavailable and so we are unsure as to how the concentrations used in this paper relate to potential *in vivo* levels. Given the lack of details regarding plasma levels of mangostin, we did not use doses greater than 100μM. However, we observed a dose dependent effect of mangostin on lagtime, TBARS, electrophoretic mobility and α-tocopherol decay and would expect larger doses to have a greater effect. The present paper examined the effects of mangostin primarily on Cu^{2+} induced peroxidation of LDL *in vitro*. This method may be appropriate to events occurring within the atherosclerotic lesion given that 'catalytic' copper ions have been reported in advanced human atherosclerotic lesions.[20]

In conclusion, this is the first paper showing that mangostin, extracted from the tropical fruit mangosteen, is an effective inhibitor of LDL peroxidation *in vitro* as indicated by lagtime to oxidation and malondialdehyde production. Derivatives of the parent compound have been produced and may offer further benefits and therapeutic potential.

References

1. D. Steinberg, S. Parthasarathy, T.E. Carew, J.C. Khoo and J.L. Witztum (1989) Beyond Cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *New England Journal of Medicine*, **320,** 915–924.
2. J.L. Witztum and D. Steinberg (1991) Role of oxidized low density lipoprotein in atherogenesis. *Journal of Clinical Investigation*, **88,** 1785–1792.
3. M.S. Brown and J.L. Goldstein (1983) Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annual Review of Biochemistry*, **52,** 223–261.
4. T. Kita, Y. Nagano, M. Yolode, K. Ishii, N. Kume, A. Ooshima, H. Yoshida and C. Kawai (1987) Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. *Proceedings of the National Academy of Science USA*, **84,** 5928–5931.
5. I. Bjorkhem, A. Henricksson-Freyschuss, O. Breuer, V. Diczfalusy, L. Bergland and P. Henrikson (1991) The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arteriosclerosis Thrombosis*, **11,** 15–22.
6. S. Parthasarathy, S.G. Young, J.L. Witztum, R.C. Pittman and D. Steinberg (1986) Probucol inhibits oxidative modification of low density lipoprotein. *Journal of Clinical Investigation*, **7,** 641–644.
7. H. Esterbauer, H. Puhl, M. Dieber-Rotheneder, G. Waeg and H. Rabl (1991) Effects of antioxidants on oxidative modification of LDL. *Annals of Medicine*, **23,** 573–581.
8. H. Mangiapane, J. Thomson, A. Salter, S. Brown, G.D. Bell and D.A. White (1992) The inhibition of the oxidation of low density lipoprotein by (+)-catechin, a naturally occurring flavonoid. *Biochemical Pharmacology*, **43,** 445–450.
9. F. Shahidi and P.K.J.P.D. Wanasundara (1992) Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition*, **32,** 67–103.
10. E.N. Frankel, J. Kanner, J.B. German, E. Parks and J.E. Kinsella (1993) Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet*, **341,** 454–457.
11. W. Mahabusarakum, P. Winiyachitra and S. Phongpaichit (1986) Antimicrobial activities of chemical constituents from *Garcinia mangostana* L.. *Journal of the Science Society of Thailand*, **12,** 239–242.
12. P. Yates and G.H. Stout (1958) The structure of mangostin. *Journal of the American Chemical Society*, **80,** 1691–1700.
13. K.D. Croft, J. Proudfoot, C. Moulton and L.J. Beilin (1991) The effect of lipoproteins on the release of some eicosanoids by stimulated human leukocytes. *Eicosanoids*, **4,** 75–81.
14. H. Esterbauer, G. Striegl, H. Puhl and M. Rotheneder (1989) Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radical Research Communications*, **6,** 67–75.
15. K.D. Croft, S.B. Dimmitt, C. Moulton and L.J. Beilin (1992) Low density lipoprotein composition and oxidisability in coronary disease – apparent favourable effect of beta-blockers. *Atherosclerosis*, **97,** 123–130.

16. B. Frei and J.M. Gaziano (1993) Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation. *Journal of Lipid Research*, **34,** 2135–2145.
17. O.H. Lowry, N.J. Roseborough, A.L. Farr and R.J. Randall (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193,** 265–275.
18. I. Jialal, G.L. Vega, S.M. Grundy (1990) Physiologic levels of ascorbate inhibit the oxidative modification of low density lipoprotein. *Atherosclerosis*, **82,** 185–191.
19. M. Thompson, C.R. Williams and G.E.P. Elliott (1976) Stability of flavonoid complexes of copper (II) and flavonoid antioxidant activity. *Analytica Chimica Acta*, **85,** 375–381.
20. C. Smith, M.J. Mitchinson, O.I. Aruoma and B. Halliwell (1992) Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochemistry Journal*, **286,** 901–905.

Accepted by Professor B. Halliwell