Inhibition of Endothelial Cell-Mediated Oxidation of Low-Density Lipoprotein by Rosemary and Plant Phenolics

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Plant-derived phenolic compounds possess antioxidant activity that may be applicable to such diverse areas as human health and the preservation of food lipids. The potential antioxidant activities of a variety of plant phenolics were investigated using human aortic endothelial cells (HAEC) to mediate the oxidation of low-density lipoprotein (LDL). Carnosic acid, carnosol, and rosmarinic acid, present in rosemary extract, and thymol, carvacrol, and zingerone, present in thyme, origanum, and ginger, were individually incubated with HAEC and LDL for 12 h. The extent of oxidation was determined spectrophotometrically by measuring absorbance (at 234 nm) of conjugated dienes. All antioxidants produced dose-dependent inhibition of LDL oxidation. Their relative antioxidant activities decreased in the order carnosol > carnosic acid ≈ rosmarinic acid >>> thymol > carvacrol > zingerone. The antioxidant activity of these plant phenolics in the HAEC system suggests that they may have potential benefits in human health.

Keywords: *LDL oxidation; rosemary; plant phenolics; human aortic endothelial cells; antioxidants*

INTRODUCTION

Lipid oxidation and its prevention by antioxidants is a subject of concern to diverse fields of research, particularly nutrition of food lipids and biomedicine. Beyond the classical antioxidants vitamins E and C, plant-derived phenolic compounds have been investigated for their antioxidant activity and applications in foods and biological systems. Among 30 spices tested in lard, rosemary had the greatest antioxidant activity (Chipault et al., 1952). The individual components of rosemary, carnosol, carnosic acid, and rosmarinic acid (Figure 1) have been evaluated for their antioxidant activities in lard, linoleic acid in emulsion (Nakatani and Inatani, 1984; Farago et al., 1989), and liposomal and microsomal systems (Aruoma et al., 1992). Likewise, the monophenols thymol, carvacrol, and zingerone (Figure 1) derived from thyme, origanum, and ginger have been assessed for their antioxidant activity in liposomal systems (Aeschbach et al., 1994). While the above reports identified the antioxidant activity of plant-derived phenolic compounds in lipid systems, their activity in biological systems is not clear. Additional assays have been developed and used to address their biological effects, including the deoxyribose assay, scavenging of hypochlorous acid, and the bleomycin assay (Farago et al., 1989; Halliwell, 1990).

The oxidative modification of low density lipoprotein (LDL) is currently viewed as a pivotal step in the pathogenesis of atherosclerosis (Esterbauer et al., 1992; Parthasarathy and Rankin, 1992; Steinberg et al., 1989; Carpenter et al., 1991). Although the exact mechanism of initiation of oxidation is not known, LDL can be oxidized in vitro by several cells of the vascular system,

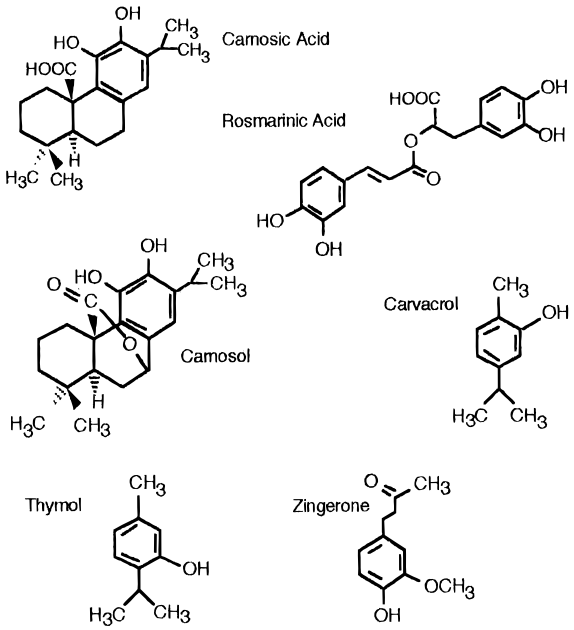

Figure 1. Structures of carnosic acid, carnosol, rosmarinic acid, thymol, carvacrol, and zingerone.

including endothelial cells (Henriksen et al., 1981; Steinbrecher et al., 1984; Morel et al., 1984), smooth muscle cells (Morel et al., 1984; Heinecke et al., 1984), and monocytes and macrophages (Morel et al., 1983; Parthasarathy et al., 1986). The modified LDL is taken up by macrophages via the scavenger receptor. This receptor is not down regulated in response to intracellular cholesterol, leading to the formation of lipid-engorged foam cells. Cell-mediated oxidation produces a minimally modified LDL particle that is similar to a subset of LDL isolated from human plasma (Cazzolato et al., 1991) and is recognized by scavenger receptors. In contrast, copper-catalyzed oxidation of LDL, commonly used to evaluate antioxidants, produces a significantly oxidized LDL particle. In this paper, the plant phenolics carnosic acid, carnosol, and rosmarinic acid, present in *Rosmarinus officinalis L.*, a crude

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rosemary extract and three monophenolics, thymol and carvacrol present in *Thymus vulgaris L.* and *Origanum vulgare L.* and zingerone present in *Zingiber officinale* (ginger), were tested with a biologically relevant system of human aortic endothelial cells (HAEC) which mediate the oxidative modification of LDL.

MATERIALS AND METHODS

Materials. HAEC were obtained from Clonetics (San Diego, CA) at passage 3. Cells were grown in Clonetics microvascular endothelial cell growth medium containing 5% fetal bovine serum, epidermal growth factor (10 ng/mL), hydrocortisone (1 μg/mL), bovine brain extract containing 10 ng/mL heparin, gentamicin (50 μg/mL), and amphotericin-B (50 ng/mL). Stock solutions of antioxidants were prepared in dimethyl sulfoxide (DMSO). Carnosol and carnosic acid were purified from rosemary extracts (Aeschbach and Phillipossian, 1990) and were greater than 98% pure by HPLC. Rosmarinic acid, thymol, and carvacrol were purchased commercially (Carl Roth GmbH, Karlsruhe, Germany). A commercial rosemary extract, obtained from FIS S.A. (Chatel-St. Denis, Switzerland) contained 10.3% carnosic acid and 4.4% carnosol by HPLC (Aruoma et al., 1992). Zingerone was obtained from ICN (Technosa, Pully, Switzerland).

Preparation of Human LDL. Blood was obtained from healthy, nonsmoking male volunteers by venipuncture into EDTA-containing vacutainer tubes. Plasma was prepared by centrifuging the blood at 1500 rpm and 4 °C. LDL was isolated by sequential density ultracentrifugation (Orr et al., 1991). EDTA was removed by dialysis overnight in pH 7.4 phosphate-buffered saline purged with nitrogen. The protein concentration in LDL was determined by the Lowry method (Lowry et al., 1951) using a Sigma protein Lowry kit (St. Louis, MO).

Cell Incubation. HAEC between passages 5 and 7 were seeded onto 35-mm six-well plates and used at confluence. The incubation media were made up immediately prior to use and consisted of phenol red-free Ham's F-10 (Gibco, Gaithersburg, MD), containing 200 μg of LDL protein/mL and the antioxidants at different concentrations of phenolics, with DMSO at a final concentration not exceeding 0.2%. Duplicate wells were washed three times in phenol red-free 37 °C Ham's F-10 to remove any growth medium and serum. The incubation media (1.2 mL) were pipetted into the wells and then incubated 12 h in a 5% CO_2 incubator. Controls consisted of cell-free wells containing the incubation medium without antioxidants and wells with HAEC containing the incubation medium with the DMSO only. Reference standards consisted of the incubation medium containing 5 μM of either α-tocopherol (Fluka Chemical Corp., Ronkonkoma, NY) or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Aldrich Chemical Co., Milwaukee, WI). The extent of lipid oxidation was determined spectrophotometrically by measuring conjugated diene hydroperoxides at 234 nm. Aliquots of each incubation medium were diluted 1:1 with Hepes phosphate buffer (pH 7.4) (Clonetics, San Diego, CA), and the baseline absorbance reading was measured at 234 nm. After a 12 h of incubation, the media were removed and centrifuged to pellet detached cells. The supernatant portion was removed and diluted 1:1 with phosphate buffer, and the absorbance was read at 234 nm. The level of conjugated dienes was determined by the change in absorbance between the zero time and 12 h readings. DMSO controls were run with each experiment. The results were calculated as percentage inhibition by the following equation: [(DMSO control − sample/DMSO control) × 100]. The three polyphenols and three monophenols were analyzed separately for significant differences between compounds and concentrations by two-way analysis of variance using the general linear model (GLM) procedures in the SAS statistical software package 6.10 (SAS Institute Inc., Cary, NC). Tukey's studentized range test ($p = 0.05$) was used to determine which combinations of compounds, and concentrations were statistically different. The concentration of phenolics and rosemary extract that inhibited cell mediated oxidation by 50% (IC_{50}) was determined by linear regression of inhibitory percentages using values of 0.3−1.25 μM for rosmarinic and carnosic acids, 0.16−1.25 μM for carnosol, 1.25−10.0 μM for carvacrol, thymol, and zingerone, and 0.3−2.5 ppm for rosemary extract.

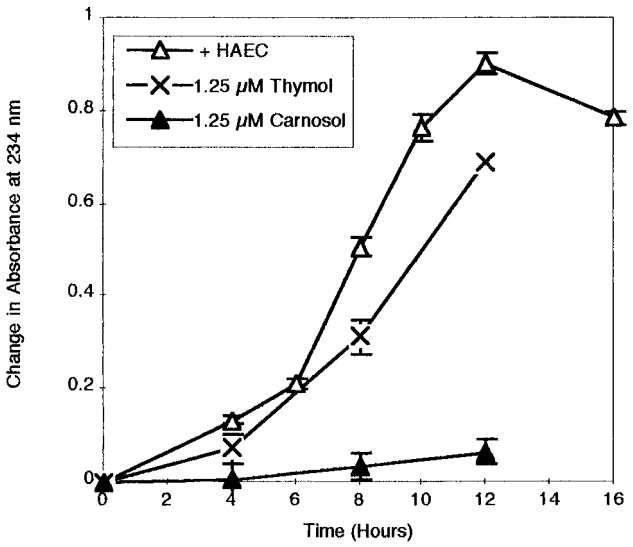

Figure 2. Time curve for conjugated diene formation from human LDL incubated with HAEC in the absence or presence of phenolic compounds. Results are expressed as the change in absorbance at 234 nm calculated as the difference between the initial absorbance (time 0 h) and the absorbance at the time points indicated, mean ± SD, $n = 2$. Data points without error bars have a standard deviation smaller than the data symbol: LDL with HAEC only (△); with HAEC and 1.25 μM thymol (×); with HAEC and 1.25 μM carnosol (▲).

RESULTS

Figure 2 shows time plots of conjugated diene formation with LDL samples incubated with HAEC. The control HAEC sample shows a lag phase of about 6 h followed by a rapid increase in conjugated dienes, reaching a maximum at 12 h, followed by a decline in absorbance. The change in absorbance between 0 and 12 h in the presence of endothelial cells reflected maximum conjugated dienes produced during LDL oxidation. Thus a 12 h incubation was chosen for testing the compounds. The addition of 1.25 μM thymol or carnosol produced decreases in conjugated diene formation (Figure 2).

Conjugated diene lipid hydroperoxides are formed from the oxidation of polyunsaturated fatty acids (PUFA). The PUFA content of LDL used in this system varied from 33 to 47% PUFA, (Frankel et al., 1992). The Ham's F-10 media used in the experiments did not contain any fetal bovine serum, linoleic acid, or other polyunsaturated fatty acids. Thus the measurement of conjugated dienes was a specific marker for LDL oxidation in this system. In the absence of endothelial cells, minimal oxidation of LDL was observed. DMSO inhibited HAEC-mediated LDL oxidation by approximately 10%. Therefore, conjugated diene production in the presence of DMSO was used to calculate percentage inhibition for the various test compounds.

The rosemary extract was very effective in inhibiting cell-mediated conjugated diene formation in a dose-dependent manner (Figure 3). From 0.3 to 2.5 ppm, rosemary extract increased inhibition of LDL linearly, followed by a plateau between 2.5 and 15 ppm at approximately 89%.

LDL oxidation was inhibited by carnosic acid, carnosol, and rosmarinic acid in a dose-dependent manner at concentrations ranging from 0.31 to 2.5 μM (Table

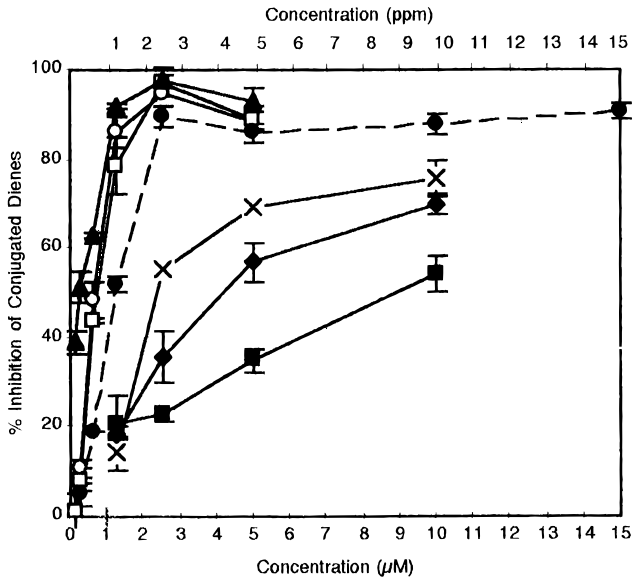

Figure 3. Relative antioxidant activities of plant phenolics and rosemary extract. Human LDL was incubated with HAEC in Ham's F-10 containing carnosol (▲), carnosic acid (□), rosmarinic acid (○), thymol (×), carvacrol (◆), zingerone (■), or rosemary extract (●). Rosemary extract is shown with a slashed line, and the units of concentration are in ppm (top *x* axis). Initial absorbance readings at 234 nm were taken and at the end of a 12 h incubation period. Results are expressed as mean % inhibition ± SD, $n = 2$. Data points without error bars have a standard deviation smaller than the data symbol.

Table 1. Inhibition of Cell-Mediated LDL Oxidation by Plant Polyphenolics[a]

concentration (μM)	% inhibition of conjugated dienes[b]		
	carnosol	carnosic acid	rosmarinic acid
0.16	$39.1 \pm 3.8^{c,x}$	$1.2 \pm 0.6^{d,y}$	nd
0.31	$51.5 \pm 3.4^{bc,x}$	$8.1 \pm 2.6^{d,y}$	$10.8 \pm 4.8^{c,y}$
0.63	$63.1 \pm 0.5^{b,x}$	$43.9 \pm 0.5^{c,y}$	$48.5 \pm 3.8^{b,y}$
1.25	$91.8 \pm 0.5^{a,x}$	$78.4 \pm 6.5^{b,x}$	$86.2 \pm 2.1^{a,x}$
2.50	$97.6 \pm 3.2^{a,x}$	$97.3 \pm 1.6^{a,x}$	$94.5 \pm 4.1^{a,x}$
5.00	$93.2 \pm 2.6^{a,x}$	$89.3 \pm 2.5^{ab,x}$	$88.4 \pm 3.5^{a,x}$

[a] Human LDL was incubated with HAEC in Ham's F-10 media in the presence or absence of the phenolic compounds. Absorbance readings at 234 nm were taken initially and at the end of a 12 h incubation period. [b] Mean % inhibition ± SD, $n = 2$. % inhibition of conjugated dienes = $\{[(C - S)/C] \times 100\}$, where C = conjugated diene production in the presence of DMSO and S = conjugated diene production in the presence of the test compound. Within columns, means with the same superscripts a−d are not significantly different ($P \leq 0.05$). Within rows, means with the same superscripts x−z are not significantly different. nd: not determined.

1). Rosmarinic acid at 0.16 μM was not tested. The antioxidant activities of carnosic acid and rosmarinic acid were equivalent at the five concentrations tested (0.31−5.0 μM) (Table 1). Carnosol was a significantly better ($P < 0.05$) antioxidant at the lower concentrations tested (0.31−0.6 μM) than were carnosic or rosmarinic acids and was significantly better than carnosic acid at 0.16 μM (Table 1). At each of the three highest concentrations tested (1.25−5.0 μM), the three polyphenolic compounds were not significantly different in antioxidant activity (Table 1).

The antioxidant activities of the monophenols, thymol, carvacrol, and zingerone, were also dose-dependent, but the concentrations needed to produce 14−75% inhibition were between 1.25 and 10 μM (Table 2). In contrast, the rosemary polyphenols had equivalent or higher antioxidant activities at much lower concentrations (Figure 2). At the lowest concentration tested (1.25 μM),

Table 2. Inhibition of Cell-Mediated LDL Oxidation by Plant Monophenolics[a]

concentration (μM)	% inhibition of conjugated dienes[b]		
	thymol	carvacrol	zingerone
1.25	$14.3 \pm 3.9^{c,x}$	$18.7 \pm 1.4^{d,x}$	$21.0 \pm 6.3^{c,x}$
2.50	$55.5 \pm 1.0^{b,x}$	$35.9 \pm 6.4^{c,y}$	$23.0 \pm 1.5^{bc,z}$
5.00	$69.6 \pm 0.5^{a,x}$	$56.9 \pm 4.3^{b,y}$	$35.0 \pm 2.6^{b,z}$
10.00	$75.7 \pm 3.9^{a,x}$	$70.0 \pm 2.4^{a,x}$	$54.3 \pm 3.9^{a,y}$

[a] See Table 1, footnote *a*. [b] See Table 1, footnote *b*.

Table 3. Concentration for 50% Inhibition of Cell-Mediated LDL Oxidation by Plant Phenolics[a]

test compounds	IC_{50}[b] (μM)
rosemary extract	1.35[c]
carnosol	0.33
rosmarinic acid	0.74
carnosic acid	0.81
thymol	4.02
carvacrol	5.53
zingerone	8.91

[a] See Table 1, footnote *a*. [b] The concentration of phenolic that inhibited cell mediated oxidation by 50% (IC_{50}) was determined by linear regression of inhibitory percentages. [c] The rosemary extract IC_{50} is expressed in ppm.

all three monophenols were not statistically different in antioxidant activity (Table 2). At the intermediate concentrations (2.5−5.0 μM), thymol had significantly greater antioxidant activity than carvacrol and carvacrol had significantly greater antioxidant activity than zingerone. Both thymol and carvacrol had significantly greater antioxidant activity than zingerone at 10 μM (Table 2). Below 1.25 μM, the monophenols exhibited no antioxidant activity. In this LDL system, the relative antioxidant ranking of these three monophenols at concentrations between 2.5 and 5.0 μM decreased in the order thymol > carvacrol > zingerone (Table 2).

The concentrations for 50% inhibition obtained with the phenolic compounds tested are summarized in Table 3. Carnosol was the most effective antioxidant among the polyphenolic compounds, followed by rosmarinic and carnosic acids. The monophenolic compounds were much less effective antioxidants, with thymol being the most active followed by carvacrol and zingerone. The rosemary extract was calculated to have an IC_{50} of 1.35 ppm, which contains 0.18 μM carnosol and 0.42 μM carnosic acid.

DISCUSSION

Plant flavonoid phenolics prevent oxidation of human LDL in vitro (DeWhalley et al., 1990; Teissedre et al., 1996) and may contribute to the prevention of atherosclerosis (Steinberg et al., 1989). Epidemiological studies showed that the intake of flavonols and flavones was inversely associated with coronary heart disease in an elderly population (Hertog et al., 1993) and in a cross-cultural population (Hertog et al., 1995). Quercetin glucosides from onions were recently shown to be absorbed and eliminated slowly in a controlled study with two human subjects (Hollman et al., 1996).

Oxidation is a complex, multistep process affecting a variety of both lipid and nonlipid compounds. Human health and disease and food quality are greatly impacted by this process. Determination of a potential antioxidant requires a variety of test systems, particularly since a substance exhibiting an antioxidant effect in one system can have a prooxidant effect in another (Laughton et al., 1989; Frankel et al., 1994).

Relative antioxidant potencies are also affected by the test system (Frankel et al., 1994; Porter et al., 1989). Frankel et al. (1994) postulated that interfacial properties of antioxidants may explain some of these differences. Accordingly, polar antioxidants are more active in bulk oil where they are proposed to be oriented at the air−oil interface. The less polar antioxidants are more protective in emulsions by being oriented at the oil−water interface, whereas the more polar antioxidants partition in the water phase. In the present study, carnosol was a stronger antioxidant than rosmarinic and carnosic acids. In accordance with the postulate of Frankel et al., carnosol being less polar more favorably partitions at the lipoprotein−water interface providing more protection, whereas the more polar rosmarinic and carnosic acids partition more into the water phase. These results are in agreement with previous results that showed the polar rosmarinic acid and carnosic acid were more active in bulk corn oil than in the corresponding corn oil emulsion, while the less polar carnosol was more active in the emulsion (Frankel et al., 1996a,b).

In the HAEC-LDL oxidation system used in the present study, carnosol exhibited greater antioxidant activity than carnosic acid. These results are in contrast to those obtained with an ox brain phospholipid liposome system (Aruoma et al., 1992). Using two different measures of liposomal oxidation, the thiobarbituric acid test and fluorescence detection, carnosic acid was more effective in inhibiting oxidation, followed by carnosol. On the other hand, in lard, on the basis of the Rancimat method (Chen et al., 1992), both carnosol and carnosic acid similarly increased the induction time. Evaluation of antioxidant activity at elevated temperatures, as in the Rancimat method, is questionable though because of artifactual results. Rosemary antioxidants decompose at elevated temperatures and produce compounds that have antioxidant activity (Schwarz et al., 1992). In rat liver microsomes, carnosol was slightly more effective in inhibiting oxidation than carnosic acid (Aruoma et al., 1992). Carnosol and carnosic acid at 5 μM inhibited microsomal oxidation by 95 and 92%, respectively. Carnosol and carnosic acid at 5.0 μM each inhibited LDL oxidation by approximately 91% (Table 1).

Of the various biologically relevant assays used to test rosemary phenolics as antioxidants, only the bleomycin assay, which detects the ability of a compound to damage DNA, yielded results suggesting adverse biological effects (Aruoma et al., 1992). However, it is difficult to relate this test, which measures carbohydrate oxidation by thiobarbituric acid reactive substances, with a measure of lipid oxidation used in this study. The inhibitory effects of rosemary phenolics for LDL oxidation are likely due to different mechanisms than those stimulating DNA damage. Nonetheless, the levels which stimulated DNA damage were between 10 and 2000 μM of carnosol and carnosic acid (Aruoma et al., 1992). These levels are 4−800-fold greater than the concentrations that produced 80−97% inhibition of LDL oxidation. These results suggest that the antioxidant activity of carnosol and carnosic acid could be attained in vivo while avoiding higher levels that may have adverse effects. Further in vivo testing is needed to clarify these effects.

The antioxidant activity of the monophenols was considerably weaker than that of rosemary phenols. In this HAEC-LDL oxidation system, the relative antioxidant activity decreased in the order thymol > carvacrol > zingerone. In an ox brain phospholipid peroxidation system, the same relative ranking was found (Aeschbach et al., 1994). The less polar thymol and carvacrol may more favorably partition at the oil−water interface of the LDL, thus providing more protection than zingerone, which is more polar and may partition more into the water phase. Similar trends were also found between this HAEC-LDL system and the corn oil systems (Frankel et al., 1995). The polar zingerone was more active than the less polar thymol or carvacrol in bulk corn oil than the corresponding emulsion, whereas thymol and carvacrol were more active in the corn oil-in-water emulsion (Frankel et al., 1995).

In the bleomycin assay, unlike carnosol and carnosic acid, thymol, carvacrol, and zingerone did not stimulate DNA damage (Aeschbach et al., 1994). Nonetheless, the concentrations needed to exert effective antioxidant activity may be difficult to obtain in physiological systems.

The individual phenolic compounds and the rosemary crude extract each inhibited LDL oxidation in this HAEC culture system, in a dose-dependent manner. Their relative antioxidant activities decreased in the order carnosol > carnosic acid ≈ rosmarinic acid >>> thymol > carvacrol > zingerone. As expected, the three polyphenols were much better antioxidants than the three monophenols. The less polar compounds of each group, carnosol, and thymol and carvacrol, were better antioxidants than the more polar carnosic and rosmarinic acids and zingerone, respectively. The use of an HAEC-LDL oxidation system may be considered as a biologically relevant means to assess the effect of antioxidants on the oxidative susceptibility of LDL particles. The antioxidant activity of the plant phenolics tested in this HAEC-LDL system suggest that they may have potential benefits in human health and disease.

ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; GLM, general linear model; HAEC, human aortic endothelial cells; LDL, low-density lipoprotein; PUFA, polyunsaturated fatty acids.

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