JPP Journal of Pharmacy and Pharmacology

JPP 2004, 56: 743–748

Received December 2, 2003
Accepted March 8, 2004
DOI 10.1211/0022357023501
ISSN 0022-3573

Acteoside protects endothelial cells against free radical-induced oxidative stress

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Abstract

The protective effect of acteoside against membrane lipid oxidation and free radical-mediated impairment of endothelial function was investigated. Results showed that iron-mediated oxidative modification of the cell membrane in cultured bovine pulmonary endothelial cells (PAECs) was significantly attenuated by acteoside as measured by thiobarbituric acid-reactive substances (TBARS). Fenton's reagent (H_2O_2/Fe^{2+}) was used to generate hydroxyl radicals (·OH) and induce oxidative stress. Acteoside not only effectively minimized the loss of cell viability induced by hydroxyl radicals in cultured endothelial cells but also countered the free radical-induced destruction of the endothelium-dependent relaxation to acetylcholine in rat aorta. Furthermore, acteoside showed a dose-dependent scavenging effect of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals and appeared to be the most efficient in comparison with the four reference compounds (α-tocopherol, vitamin C, probucol and resveratrol). These data suggested that acteoside protects the cell from oxidative stress and that scavenging of free radicals could be a key mechanism contributing to the cytoprotective effect of acteoside.

Introduction

Oxygen free radicals have been implicated in the pathophysiology of various vascular disease states (Prasad & Kalra 1993). Vascular endothelial cells are vulnerable to free radicals because of their location in the vasculature. Direct mechanical damage of endothelial cells results from peroxidation of cellular lipid and proteins. Endothelial cell functional damage results from free radical inactivation of endothelium-derived relaxing factor, nitric oxide (NO) (Rubanyi & Vanhoutte 1986). Therefore, both cellular anatomical disruption and inactivation of NO by these radicals result in the loss of vascular regulation. The administration of free radical scavengers and antioxidants has been shown to provide protection against endothelial cell damage.

Much attention has been focused on identifying dietary factors capable of inhibiting oxidative modification of membrane lipid. Recent biochemical studies have described the hierarchy of the antioxidant potential of polyphenols against radicals generated in the aqueous phase and there are many reports of their inhibitory effects in lipid systems (Terao et al 1994; Rice-Evans et al 1995). In particular, catechins (polyphenolic constituents of tea), quercetin, kaempferol and anthocyanidins (constituents of grape skin and red wine) have been shown to be effective antioxidants against radicals in the aqueous and lipophilic phases (Mangiapane et al 1992; Renaud & de Lorgeril 1992; Rice-Evans et al 1995). However, little attention has been focused on the antioxidant activity of simple phenolic acids or phenylpropanoids present in the diet, which are at higher concentrations than the polyphenolic flavonoids and anthocyanidins (Wang et al 1996).

Various plants used in traditional medicine contain significant amounts of phenylpropanoids (Ismailoglu et al 2002). For example, acteoside (2-(3,4-dihydroxyphenylethyl)-1-*O*-α-L-rhamnopyranosyl-(1-3)-β-D-(4-*O*-caffeyl)-glucopyranoside) is a phenypropanoid glycoside widely distributed in plants (Pu et al 2003). Many studies have reported that acteoside shows anti-inflammatory (Sahpaz et al 2002), anti-nephritic (Hayashi et al 1994) and anti-hepatotoxic activity (Xiong et al 1998). Recently, we also isolated acteoside from a Chinese herbal medicine, *Orobanche caerulescens*, and demonstrated that this compound acts as an antioxidant (Lin et al 2004). Acteoside

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Funding: This work was supported by National Research Institute of Chinese Medicine, Taipei, Taiwan, R.O.C.

contains a hydroxyphenylethyl and a caffeoyl moiety, which are well-known antioxidants. This antioxidative function would be of benefit given that acteoside is used in the prevention of cardiovascular disorders such as atherosclerosis. In this study, we determined whether acteoside scavenges free radicals already generated or whether it prevents free radical-induced impairment of vascular endothelial function.

Materials and Methods

Isolation of acteoside from *Orobanche caerulescens*

The ethanol extract of *O. caerulescens* was subjected to Diaion HP-20 column chromatography as described by Lin et al (2004). After repeated chromatography, acteoside was isolated. The purity, as determined by HPLC with an UV detector (280 nm), was greater than 99.8%.

Culture of endothelial cells

Bovine pulmonary artery endothelial cells (PAECs) (American Type Culture Collection, Rockville, MD) were grown in EMEM (Gibco, USA) and supplemented with 20% fetal bovine serum (FBS), 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids and 1.5 g L^{-1} sodium bicarbonate. Cells were verified by typical cobblestone morphology under a microscope and were used between the 7th and 10th passages.

Cell morphology
After culture in EMEM for 18 h, with or without $FeSO_4$, cells were fixed by adding 4% paraformaldehyde (Sigma). Cell morphology, in terms of changes in cell perimeter and cell spreading, was examined through an inverted light microscope.

Measurement of membrane lipid oxidation by TBARS assay

PAECs were pre-treated with acteoside for 30 min, then membrane lipid oxidation was induced by incubation with a high concentration (50 μM) of $FeSO_4$ for 18 h at 37°C. The extent of membrane lipid oxidation was determined by the thiobarbituric acid-reactive substances (TBARS) method and the absorbance was determined at 535 nm against a blank that contains all the reagents minus sample (Areias et al 2000). Malondialdehyde (MDA) obtained by acidification of malonaldehyde-bis-dimethyl-acetal (Aldrich, USA) was used as the standard for the quantification of TBARS. Results are expressed as nmol MDA/mg protein.

Induction of free radical-mediated cell injury and analysis of cell viability

The hydroxyl radical (•OH) is one of the strongest oxidants and can be produced by Fenton's reagent (H_2O_2/Fe^{2+}) (Walling 1975). Briefly, PAEC cells (2×10^5 cells/well) in 96-well plates were incubated with various concentrations of acteoside for 30 min at 37°C. Oxidative stress was then initiated by the co-addition of H_2O_2 (1 or 2 mM) and 10 μM $FeSO_4$ and carried out for another 2 h. Cell viability was assessed by the mitochondria-dependent reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan.

Preparation of aortic rings and induction of free radical-induced vascular endothelium dysfunction

All experiments were performed in accordance with the Guidelines for Animal Experiments of the National Research Institute of Chinese Medicine. Thoracic aorta obtained from male Sprague-Dawley rat (250–300 g) was cut into rings and mounted in an organ bath filled with oxygenated (95% O_2/5% CO_2) Krebs' solution (Chiou et al 1998). After the aortic rings were randomized to receive acteoside for 15 min, Fenton's reagent was added to the organ bath for 30 min to induce free radical injury (Tsai et al 2003). Sub-maximal contraction was achieved by addition of 0.3 μM phenylephrine (Sigma) and cumulative relaxation curves to acetylcholine (Sigma) were obtained to assess endothelial function.

DPPH scavenging assay

The free radical scavenging activity of acteoside was tested by employing the 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma) radical scavenging assay. Briefly, to a solution of DPPH (final concentration 200 μM) in absolute ethanol, an equal volume of the compound dissolved in ethanol was added at various concentrations. Ethanol was added to the control solution. Absorbance was recorded at 518 nm at room temperature (Andreadou et al 1997). Each experiment was performed at least in triplicate.

Statistical analysis

All values in the text and figures represent means ± s.e.m. of n observations. Data were analysed by a two-way analysis of variance. The effect of increasing the concentration of acteoside and the presence or absence of the hydrogen peroxide was evaluated using post-hoc Tukey's test. $P < 0.05$ was considered statistically significant.

Results

Effect of acteoside on cell morphology

After 18 h incubation in culture medium, control PAECs displayed a characteristic morphology of cytoplasmic spreading with about 90% of the cells showing a high degree of polarity and the average cell perimeters appearing elongated (Figure 1). The presence of a high concentration of $FeSO_4$ (50 μM) for 18 h resulted in major morphological cellular changes — about 99% of the cells were spherical in shape (Figure 1) with some cells being

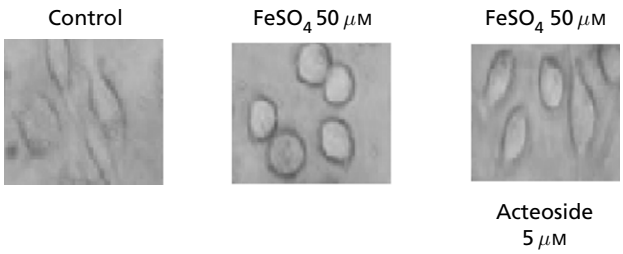

Figure 1 Effects of $FeSO_4$ on cell morphological change before and after acteoside pre-treatment in cultured bovine pulmonary arterial endothelial cells. Cells were pre-treated with acteoside (5 μM) for 30 min. After culture in medium, with or without $FeSO_4$, for another 18 h, cells were fixed by adding 4% paraformaldehyde. Cell morphology, in terms of changes in cell perimeter and cell spreading, was examined through an inverted light microscope.

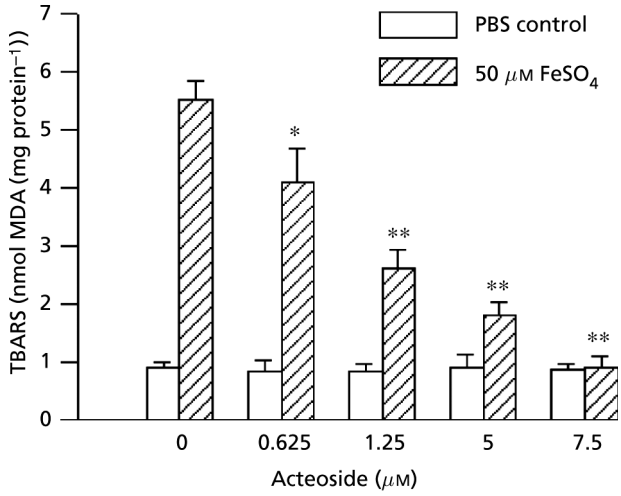

Figure 2 Effect of acteoside on $FeSO_4$-mediated membrane lipid oxidation in cultured bovine pulmonary arterial endothelial cells as determined by the thiobarbituric acid-reactive substances (TBARS) method. Cells were pre-treated with acteoside for 30 min and then incubated with $FeSO_4$ (50 μM) for another 18 h. All values are mean $\pm$ s.e.m. of triplicate samples from five independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ compared with samples exposed to $FeSO_4$ alone.

detached from the bottom of the wells (data not shown). In the absence of $FeSO_4$, acteoside alone did not significantly modify cell shape (data not shown). However, acteoside displayed a beneficial effect against iron-induced morphological change — PAECs pre-treated with acteoside tended to show cytoplasmic spreading and elongation (Figure 1). Such effects also appeared to be concentration-dependent (data not shown).

Effect of acteoside on membrane lipid oxidation

We further evaluated the protective effects of acteoside against membrane lipid oxidation induced by iron. No significant change in TBARS background level was noted when acteoside alone was incubated with PAECs (Figure 2 (open bars)). Administration of 50 μM $FeSO_4$ for 18 h induced an approximately 5.5-fold increase in TBARS values. When cells were pre-treated with acteoside (0.625–7.5 μM), a concentration-dependent reduction in TBARS formation was observed (Figure 2) with a significant effect being noted even at a concentration as low as 0.625 μM ($P < 0.05$).

Cytotoxicity

Alterations in cellular viability decreased MTT production. We showed that incubation of PAECs with 1 and 2 mM H_2O_2 (in the continuous presence of 10 μM $FeSO_4$) evoked a striking decrease in the cell viability (by 27% and 46%, respectively) (Figure 3). Acteoside alone did not induce any cellular toxicity but concentration-dependently reversed the cell death caused by Fenton's reagent (H_2O_2/Fe^{2+}).

Effect of acteoside on free radical-mediated impairment of endothelium-dependent vasorelaxation

In control aortic rings, phenylephrine produced 1.7 ± 0.4 g of contractile force. A lower concentration of H_2O_2 (0.5 mM) was chosen to induce appropriate free radical injury. Under this condition, endothelium-dependent vasorelaxation to acetylcholine was destroyed but the vasoconstriction to phenylephrine was not affected significantly (1.5 ± 0.3 g). As shown in Figure 4, endothelium-dependent vasodilatation to acetylcholine ($0.01 \sim 10$ μM) was significantly impaired after exposure to $H_2O_2/FeSO_4$ when compared with control vessels. There was no difference in endothelium-independent vasodilatation to sodium nitroprusside (0.1 μM) between control vessels and those exposed to $H_2O_2/FeSO_4$ (97.2 ± 6.3 vs 98.1 ± 5.5%). Acteoside alone, at the concentrations used (1.25 and 5 μM), did not modify the basal vascular tone, the vasoconstriction response to phenylephrine or the original vasorelaxant response to acetylcholine, neither did it produce direct vasodilatation (data not shown). However, pre-treatment of the vessels with acteoside 30 min before $H_2O_2/FeSO_4$ caused an apparent concentration-dependent reversal of vascular hyporeactivity to acetylcholine (Figure 4).

Effect of acteoside on DPPH absorbance

The radical scavenging activity of acteoside was estimated by reactivity with DPPH. The absorbance of DPPH control did not change obviously for 180 min in the absence of antioxidants. However, DPPH decolorization was significantly increased by acteoside in a concentration-dependent manner, with an IC50 of 3.4 ± 0.9 μM (Figure 5A). After 180 min, the percent of DPPH reduced reached 85.2 ± 0.3% in the presence of 5 μM acteoside. When 50 μM acteoside was added to the mixture, the absorbance decreased quickly and reached a steady state after 1 min; the percent of DPPH reduced was 91.4 ± 1.1% after 180 min. We also evaluated the interaction of four reference antioxidant substances with DPPH and compared with acteoside. Figure 5B showed

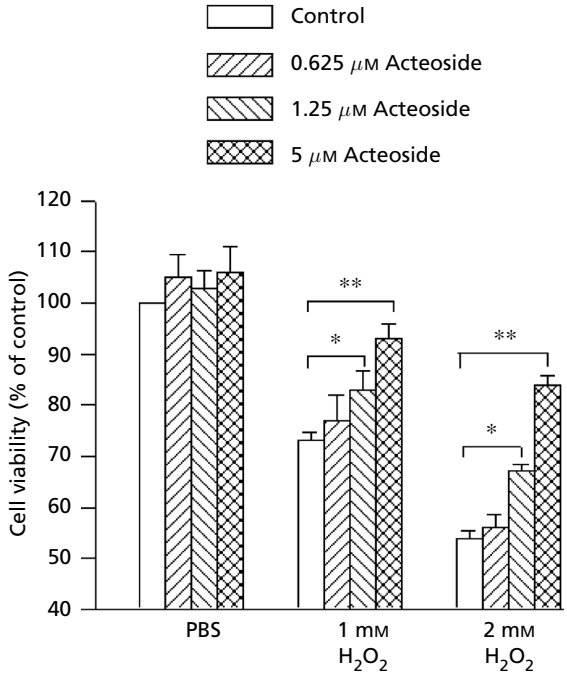

Figure 3 Effect of acteoside on H_2O_2/Fe^{2+}-induced cytotoxicity in cultured bovine pulmonary arterial endothelial cells. Cells were pre-incubated with acteoside for 30 min, and then exposed to H_2O_2 (1 and 2 mM) in the continuous presence of $FeSO_4$ (10 μM) for 2 h. Cell viability was measured by the MTT assay. All values are mean ± s.e.m. of triplicate samples from five independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ compared with samples exposed to H_2O_2/Fe^{2+} alone.

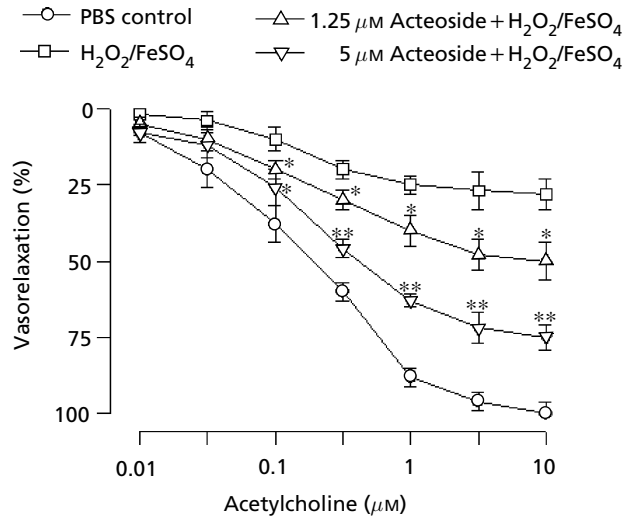

Figure 4 Effect of acteoside on H_2O_2/Fe^{2+}-mediated impairment of endothelium-dependent relaxation to acetylcholine in rat aortic rings pre-contracted with phenylephrine. Vascular rings were pre-incubated with acteoside for 30 min then exposed to H_2O_2 (0.5 mM) in the continuous presence of $FeSO_4$ (10 μM). All values are mean ± s.e.m. of data from eight independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ compared with samples exposed to $H_2O_2/FeSO_4$ alone.

that resveratrol, α-tocopherol, probucol and vitamin C were all less effective than acteoside on DPPH decolorization: at a concentration of 10 μM, they only caused a moderate decrease of 42.8 ± 0.9%, 45.5 ± 0.3%, 52.9 ± 0.7% and 34.7 ± 0.6% in the absorbance of DPPH, respectively.

Discussion

There is considerable recent evidence that free radicals induce oxidative damage to biomembranes. This damage causes atherosclerosis, aging and several other diseases (Nayak et al 2001; Biesalski 2002). Antioxidants, which scavenge free radicals, are known to have an important role in preventing these free radical-induced diseases. In this study, 18 h treatment with a high concentration of iron altered cell morphology whereby cells tended to resist the cytoplasmic spreading and rounded up. This condition also successfully induced lipid oxidation of the biomembranes in cultured PAECs as measured by TBARS formation. These results demonstrated that all these phenomena could be significantly ameliorated by acteoside.

The hydroxyl radical (·OH), one of the strongest oxidants that can be generated by Fenton's reagent (H_2O_2/Fe^{2+}) (Walling 1975), induces functional endothelial injury, which can be prevented by free radical scavengers. As further evidence, we also demonstrated the impairment of acetylcholine-induced endothelium-dependent relaxation by Fenton's reagent. Our results showed that acteoside possesses protective activity against Fenton's reagent (H_2O_2/Fe^{2+})-mediated harmful effects on culture endothelial cells and prevents H_2O_2/Fe^{2+}-induced impairment of endothelium-dependent relaxation in isolated rat aorta. Wong et al (2001) demonstrated that higher concentrations of acteoside ($0.1 \sim 0.4$ mg mL^{-1}, were calculated equal to $100 \sim 600$ μM) induced direct vasorelaxing effect on U46619-precontracted rat aorta. Comparison with the results obtained by Wong et al (2001) indicated that the concentrations of acteoside (1.25 and 5 μM) used to prevent free-radical-mediated injury of vascular endothelium in our study is 100 times lower than that which evokes a direct vasorelaxing effect. By contrast, Tam et al (2002) reported that acteoside, at concentrations ranging from $3 \sim 50$ μM, enhanced phenylephrine-induced contraction without affecting the maximum response in rat mesenteric artery. An interesting finding of our study was that lower concentrations of acteoside (1.25 and 5 μM) did not modify the basal vascular tone, the vasoconstriction response to phenylephrine or the original vasorelaxant response to acetylcholine, and neither did they produce direct vasodilatation, although the vascular endothelium was effectively protected from free radical-mediated injury.

Several compounds isolated from plants, such as flavonoids, phenolic acid, phenolic diterpenes, alkaloids, chlorophyll derivatives and carotenoids, possess free radical scavenging property (Larson 1988). For example, flavonoids have a phenolic structure and their free radical scavenging activity depends on the hydrogen-donating capacity of the

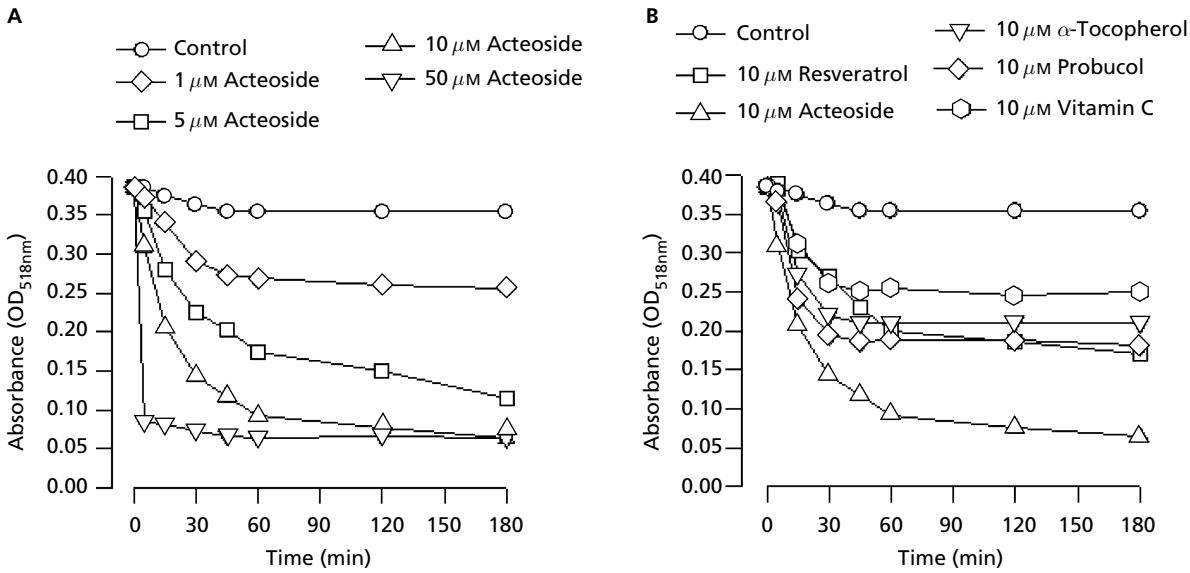

Figure 5 DPPH radical scavenging activity of acteoside (A), resveratrol (B), α-tocopherol (B), probucol (B) and vitamin C (B). All values are mean $\pm$ s.e.m. from five independent experiments.

hydroxyl groups in this structure (Saija et al 1995). It is well known that hydroxyl groups in aromatic compounds, especially those having ortho di- or trihydroxy-function, can form a stable radical and thus prevent the free radical-induced tissue injury (Rice-Evans et al 1995). Some activities of phenylpropanoid glycosides have also been suggested to be dependent on their antioxidant function. A study focused on testing many phenylpropanoid glycosides both in-vivo and in cultured keratinocytes demonstrated that they had protective potential against the effects of free oxygen radicals (superoxide and hydroxyl) (Andary 1993).

For a better understanding of acteoside's behaviour in protecting against biomembrane oxidation, as well as in reserving endothelium function, the free radical scavenging capacity of acteoside was tested by its ability to bleach the stable DPPH radical (Yamaguchi et al 1998; Sala et al 2003). During the assay, the reaction was very stable, producing reliable values in repeated tests because DPPH containing an odd electron gives a strong absorption at 518 nm in a visible spectrophotometer. As this electron becomes paired off in the presence of a free radical scavenger, the absorption fades and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. Our results showed that acteoside decreased the absorption of DPPH, hence it acted as a free radical scavenger and appeared to be the most efficient in comparison with the four reference compounds tested. Thus, we suggest that acteoside effectively protects the cell membrane from oxidative stress and that this may be related to its free radical scavenging activity.

Conclusion

In conclusion, acteoside may exert a beneficial effect on biomembranes lipid oxidation by scavenging free radicals. This concept was further supported by the DPPH test that provided information regarding the reactivity of acteoside with a stable free radical. It is well known that oxygen free radicals are involved in the pathology of many diseases. This investigation demonstrated that acteoside has a strong protective action against oxygen free radical-induced peroxidative damage to biomembranes. Additional studies will be needed to explore the potential of acteoside as a novel reagent for basic biochemical research. If shown to be safe in biological studies, this compound might be useful in the prevention or treatment of various chronic diseases associated with oxidative stress.

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