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Cancer Chemopreventive and Antioxidant Activities of Pterostilbene, a Naturally Occurring Analogue of Resveratrol

AGNES M. RIMANDO,*,† MURIEL CUENDET,‡ CRISTIAN DESMARCHELIER,§ RAJENDRA G. MEHTA,|| JOHN M. PEZZUTO,‡ AND STEPHEN O. DUKE†

Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 8048, University, Mississippi 38677; Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy (m/c 877), University of Illinois at Chicago, 833 South Wood Street, Chicago, Illinois 60612; IQUIMEFA-CONICET, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, Buenos Aires, Argentina; and Department of Surgical Oncology, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612

Pterostilbene, a natural methoxylated analogue of resveratrol, was evaluated for antioxidative potential. The peroxyl-radical scavenging activity of pterostilbene was the same as that of resveratrol, having total reactive antioxidant potentials of 237 ± 58 and 253 ± 53 μM, respectively. Both compounds were found to be more effective than Trolox as free radical scavengers. Using a plant system, pterostilbene also was shown to be as effective as resveratrol in inhibiting electrolyte leakage caused by herbicide-induced oxidative damage, and both compounds had the same activity as α-tocopherol. Pterostilbene showed moderate inhibition (IC_{50} = 19.8 μM) of cyclooxygenase (COX)-1, and was weakly active (IC_{50} = 83.9 μM) against COX-2, whereas resveratrol strongly inhibited both isoforms of the enzyme with IC_{50} values of approximately 1 μM. Using a mouse mammary organ culture model, carcinogen-induced preneoplastic lesions were, similarly to resveratrol, significantly inhibited by pterostilbene (ED_{50} = 4.8 μM), suggesting antioxidant activity plays an important role in this process.

KEYWORDS: Cyclooxygenase; mouse mammary gland organ culture; total reactive antioxidant potential; electrolyte leakage

INTRODUCTION

Pterostilbene, reported to be the only stilbene found in the genus *Pterocarpus*, was first isolated from *P. santalinus* (red sandalwood) (*1*). Together with resveratrol, it has also been identified in *Vitis vinifera* leaves (*2*), in infected grape berries var. Chardonnay and Gamay (*3*), and in healthy and immature berries var. Pinot Noir and Gamay (*4*). Pterostilbene has not been detected in wine (*5*).

Pterostilbene was found to be one of the active constituents in extracts of the heartwood of *P. marsupium*, used in Ayurvedic medicine for the treatment of diabetes (*6*). When administered to streptozotocin-induced hyperglycemic rats, pterostilbene and marsupin, two of the major phenolic constituents in aqueous decoctions of the heartwood of *P. marsupium*, significantly decreased plasma glucose level by 42 and 33%, respectively, with activities comparable to metformin (48% decrease), an oral hypoglycemic agent (*6*).

Resveratrol has been the subject of numerous research investigations. Its cancer chemopreventive activity has been demonstrated in assays representing tumor initiation, promotion, and progression of carcinogenesis (*7*). Epidemiological studies found low incidence of coronary heart disease among wine-drinking populations (*8*, *9*) correlate such observations with resveratrol present in wine. This seems to be supported by studies in rats and mice which showed that resveratrol and its glycoside piceid inhibited the accumulation of cholesterol and triglycerides in rat liver, and reduced triglyceride synthesis in mice liver (*10*). Furthermore, piceid reduced serum triglyceride and low-density lipoprotein-cholesterol levels, and atherogenic index (*10*). The antioxidant activity of resveratrol has been studied to a great extent. Resveratrol prevented Fe^{2+}-induced lipid peroxidation in rat liver microsomes (*11*, *12*), inhibited Cu^{2+}-catalyzed human low-density lipoprotein (LDL) peroxidation (*13*) and porcine LDL peroxidation in the presence as well as in the absence of $CuSO_4$ (*14*), and *tert*-butylhydroperoxide-induced lipid peroxidation in human fibroblasts (*12*). Resveratrol also inhibited the production of reactive oxygen species in porcine platelets (*15*) and in murine macrophages (*16*). The antioxidant property of resveratrol was also demonstrated through its direct scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (*12*, *17*) and inhibition of oxidation of citronellal (*12*). The demonstrated biological and pharmacological activities of resveratrol are attributed to its antioxidant property.

* Corresponding author [phone 662-915-1037, fax 662-915-1035, email arimando@ars.usda.gov].
† U.S. Department of Agriculture.
‡ College of Pharmacy, University of Illinois at Chicago.
§ University of Buenos Aires.
|| College of Medicine, University of Illinois at Chicago.

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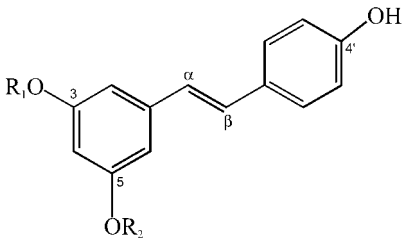

Pterostilbene, $R_1 = R_2 = CH_3$
Resveratrol, $R_1 = R_2 = H$

Figure 1. Structure of pterostilbene and resveratrol.

There is, evidently, a vast amount of research work done in evaluating the activity of resveratrol using animal and microbial systems. On the contrary, there is almost no work done to show its effects using plant systems. The physiological function of resveratrol in plants is, currently, not clearly known. One study has shown that *trans*-resveratrol inhibited plant oxidative and antioxidative enzymes, namely, superoxide dismutase, lipoxygenase, catalase, peroxidase, polyphenol oxidase, and 1-aminocyclopropane-1-carboxylic acid oxidase (*18*).

There is no work known which demonstrates the antioxidant effects of pterostilbene using a plant system. The antioxidative activity of pterostilbene was first demonstrated in vitro by its inhibition of methyl linoleate oxidation (*19*). More recently, pterostilbene was reported to scavenge DPPH free radical and to inhibit the oxidation of citronellal, and lipid peroxidation in rat liver microsomes and in cultured human fibroblasts (*12*). Pterostilbene isolated from *Anogeissus acuminata* (Fam. Combretaceae) was also found to be cytotoxic against a number of cancer cell lines including human breast cancer (ED_{50} 13.6 μM) and murine lymphoid neoplasma (ED_{50} 10.1 μM) (*20*). Methoxylated analogues of resveratrol, except pterostilbene, were synthesized and were reported to demonstrate structure−activity differences in their inhibitory activity against normal and cancerous human diploid fibroblasts, and also against preneoplastic human mammary epithelial cells (*21*).

Prompted by the close structural similarity of pterostilbene and resveratrol (**Figure 1**), as well as pterostilbene's presence in grapes and its reported cytotoxic activities, we currently report studies designed to investigate its cancer chemopreventive potential and its activity against cyclooxygenase (COX). A further objective was to investigate its antioxidant activity in comparison with that of resveratrol.

MATERIALS AND METHODS

Preparation of Pterostilbene. Pterostilbene was prepared by partial methylation of *trans*-resveratrol (Sigma-Aldrich, St. Louis, MO). To a solution of *trans*-resveratrol [150 mg in 3.0 mL of 20% (v/v) MeOH], 5 mL of 38% NaOH and 2.5 mL of dimethyl sulfate were added dropwise, with agitation, under nitrogen. The mixture was heated to 40 °C for an additional hour, and then cooled to room temperature. The partially methylated products were extracted with $CHCl_3$ and dried with Na_2SO_4. Pterostilbene was obtained by preparative TLC (developing solvent, hexane/EtOAc, 8:2, R_f 0.6), yield 11%. Its structure and identity were confirmed by comparison of ^{1}H- and ^{13}C NMR data (obtained on a Bruker Avance DPX 300 instrument; Billerica, MA) with published values (*20*). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.40 (2H, d, J = 8.50 Hz; H-2′and H-6′), 7.03 (1H, d, J = 16.20 Hz; H-β), 6.89 (1H, d, J = 16.20 Hz; H-α), 6.83 (2H, d, J = 8.51 Hz; H-3′and H-5′), 6.65 (2H, d, J = 1.88 Hz; H-2 and H-6), 6.38 (1H, t, J = 1.88 Hz; H-4), 4.96 (1H, br s; OH), 3.83 (6H, s, 2 × OCH_3). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 160.92 (C-2 and C-5), 155.31 (C-4′), 139.61 (C-1), 130.10 (C-1′), 128.63 (C-β), 127.99 (C-2′), 126.60 (C-α), 115.60 (C-3′ and C-5′), 104.31 (C-6), 99.59 (C-4), 55.35 (OCH_3).

Mouse Mammary Gland Organ Culture (MMOC) Model of Carcinogenesis. The cancer chemopreventive activity of pterostilbene was evaluated using MMOC following reported procedures (*22*). Briefly, thoracic pairs of mammary glands from 4 to 5 weeks old Balb/c female mice were incubated in serum-free medium supplemented with 5 μg/mL each of insulin and prolactin, and 1 μg/mL of aldosterone and hydrocortisone as growth-promoting hormones. Incubations were performed in the presence (10, 3, or 1 μM) or absence of pterostilbene (15 glands/group). On the third day of culture the chemical carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) was added to the culture dish and incubated for 24 h. The medium was changed the following day, and incubation was continued for 6 more days without DMBA. The glands were then incubated for 14 more days in medium containing only insulin. Finally, glands were fixed in 10% buffered formalin and stained with alum carmine. The stained glands were evaluated microscopically for the presence of mammary lesions and the incidence of mammary lesions ((number of glands with lesions/total number of glands) × 100) was calculated.

Evaluation of COX-1 and COX-2 Activity by Quantitation of Prostaglandin E_2 (PGE_2). The effect of pterostilbene and resveratrol on COX activity was determined by measuring PGE_2 production (*23*). Reaction mixtures were prepared in 100 mM Tris−HCl buffer, pH 8.0, containing 1 μM heme, 500 μM phenol, 300 μM epinephrine, sufficient amounts of COX-1 or COX-2 to generate 150 ng of PGE_2/mL, and various concentrations of test samples. The reaction was initiated by the addition of arachidonic acid (final concentration, 10 μM) and incubated for 10 min at room temperature (final volume 200 μL). The reaction was then terminated by adding 20 μL of the reaction mixture to 180 μL of 27.8 μM indomethacin, and PGE_2 was quantitated by an ELISA method. Samples were diluted to the desired concentration with 100 mM potassium phosphate buffer (pH 7.4) containing 2.34% NaCl, 0.1% bovine serum albumin, 0.01% sodium azide, and 0.9 mM Na_4-EDTA. Following transfer to a 96-well plate (Nunc-Immuno Plate Maxisorp, Fisher) coated with a goat anti-mouse IgG (Jackson Immuno Research Laboratories), the tracer (PGE_2-acetylcholinesterase, Cayman Chemical, Ann Arbor, MI) and primary antibody (mouse anti-PGE_2, Monsanto, St. Louis, MO) were added. Plates were then incubated at room-temperature overnight, reaction mixtures were removed, and wells were washed with a solution of 10 mM potassium phosphate buffer (pH 7.4) containing 0.01% sodium azide and 0.05% Tween 20. Ellman's reagent (200 μL) was added to each well, and the plate was incubated at 37 °C for 3−5 h, until the control wells yielded an OD = 0.5−1.0 at 412 nm. A standard curve with PGE_2 (Cayman) was generated on the same plate, which was used to quantify the PGE_2 levels produced in the presence of test samples. Reactions were conducted in duplicate, average values were expressed as a percentage relative to control (solvent-treated) samples, and dose−response curves were constructed for the determination of IC_{50} values. The variability of pterostilbene's IC_{50} values less than 10%.

Peroxyl Radicals ($ROO^•$) Scavenging Activity: Total Reactive Antioxidant Potential (TRAP). The TRAP index was measured by luminol-enhanced chemiluminescence (*24*). This method is based on the inhibition of the generation of peroxyl radicals by the radical cation 2,2′-azo-bis(2-amidinopropane) (ABAP), which reacts with luminol yielding chemiluminescence. The reaction medium consisted of 100 mM phosphate buffer (pH 7.4), 20 mM ABAP (Acros Organics, Janssens Pharmaceuticalaan 3A, 2440 Geel, Belgium), 10 μM luminol (Acros Organics), and increasing volumes (5−20 μL) of pterostilbene or resveratrol (150 μM). Luminescence was measured at room temperature in a WinSpectral (model 1414) liquid scintillation counter (Turku, Finland) with the circuit coincidence out of mode. The system was calibrated using 150 μM of the synthetic α-tocopherol analogue Trolox (Sigma-Aldrich). Induction times, the period during which luminescence remained reduced after addition of the samples indicating the presence of free-radical scavengers, were determined by measurement of chemiluminescence intensity (total counts). A comparison of the induction times after addition of known concentrations of Trolox and antioxidants (shown later in **Figure 3**) allows TRAP values to be obtained as equivalents of Trolox concentration necessary to suppress the emitted luminescence (*25*) by employing the following equation:

$$\text{TRAP } (\mu\text{M Trolox}) = (\mu L_{total}/\mu L_{sample}) \times (\delta_{i\ sample}/\delta_{i\ Trolox}) \times \delta_{i\ Trolox\ (1\mu M)}$$

where μL_{total} is the final volume (3000 μL), μL_{sample} is the volume of the sample added to the reaction (5, 10, 15, and 20 μL of a 150 μM solution), $\delta_{i\ sample}$ is the induction time observed for the different volumes of the sample, $\delta_{i\ Trolox}$ is the induction time observed for the reference compound (Trolox), and $\delta_{i\ Trolox\ (1\mu M)}$ is the induction time observed for the reference compound at a 1 μM final concentration. Using Trolox as a reference inhibitor that removes two radicals per added molecule (*25*), it is possible to obtain the number of radicals removed per molecule of additive.

***In vivo* Antioxidant Assay in Plant Tissue.** Cucumber seedlings (*Cucumis sativus* L. cv. Long Green Improved) were used for plant oxidative damage experiments. Seeds were planted in Miracle Gro potting soil and grown in a chamber maintained at 25 ± 2 °C with continuous 200 μmol m^{-2}•s^{-1} photosynthetically active radiation (PAR). Oxidative damage to plant tissues was monitored by measurement of herbicide-induced electrolyte leakage, as previously described (*26*). Fifty 4-mm cucumber cotyledon disks (~0.1 g fresh weight) from 6-day-old seedlings were placed in a 6-cm-diameter disposable Petri dish containing 5 mL of 1% (w/v) sucrose and 1 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES; pH 6.5). Acifluorfen (5-[2-chloro-4-(trifluoromethyl)phenoxy]2-nitro-benzoic acid; Sigma-Aldrich Laborchemikalien GmbH**,** D-30918 Seelze, Germany) was dissolved in this media to make a 10 mM stock solution. Resveratrol, pterostilbene, and α-tocopherol were dissolved in methanol, which was placed into the Petri dish and allowed to dry before the buffer media was added (control contained media only). This method was used because of the relative insolubility of these compounds in water. All treatments were performed in triplicate and the experiment was replicated in time. The Petri dishes were placed in darkness at 25 °C for 18 h and then exposed to 525 μmol m^{-2}•s^{-1} PAR for the remainder of the experiment. Cellular damage was determined by measuring electrolyte leakage in the bathing solution with a conductivity meter capable of assaying 1 mL of bathing medium (*27*). Conductivity was measured for 18 h in darkness, followed by continuous light. Because of differences in background conductivity between different treatments, results are expressed as change in the conductivity after exposure to light.

RESULTS AND DISCUSSION

Pterostilbene used in the studies reported here was synthesized in a one-step reaction by permethylaton of *trans*-resveratrol. The product obtained was identical with the naturally occurring pterostilbene as confirmed from its NMR data.

The cancer chemopreventive property of pterostilbene was investigated using a mouse mammary gland culture model of carcinogenesis. This model mimics the morphologic and biochemical changes observed in animals during various physiologic stages of the gland in transition from virgin to pregnancy and lactation, and it is the closest adaptation of the mammary gland in vivo (*22*). The entire cycle of mammary gland morphology and physiology can be simulated with appropriate hormonal supplementation of a chemically defined medium (*28*). The validity of this model has been proven in the assay of known chemopreventive agents (*29*). The glands show largely ductal structures and very few end buds when observed under the microscope. However, in the presence of the chemical carcinogen DMBA, mammary alveolar precancerous lesions develop. The incidence of mammary alveolar formation (number of glands with lesions/number of total glands in the group) is compared between treated and control groups and the percent inhibition is calculated. For pterostilbene, an ED_{50} of 4.8 μM (**Figure 2**) was obtained. The potential of resveratrol to function as a cancer chemopreventive agent was previously evaluated using this system and was found to inhibit preneoplastic lesions induced by DMBA with an ED_{50} of 3.2 μM (*7*). It appeared

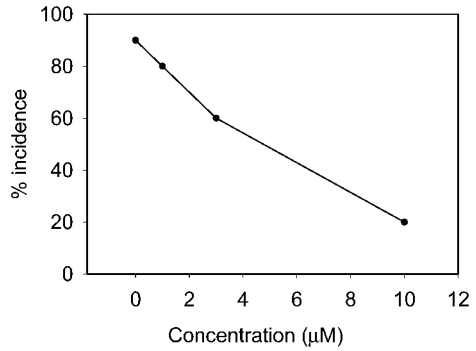

Figure 2. Effect of pterostilbene on the development of DMBA-induced mammary alveolar lesions in organ culture. Data from groups treated with pterostilbene were compared with those from control (DMBA only) groups and results were expressed as a percentage.

that pterostilbene has cancer chemopreventive activity similar to that of resveratrol. The activity of pterostilbene against COX-1 and -2 was then further investigated, because COX-1 and -2 enzymes are implicated in the tumor promotion stage of carcinogenesis (*23*, *29*). Pterostilbene was found to only moderately inhibit COX-1 (IC_{50} 19.8 μM) and weakly inhibit COX-2 (IC_{50} 83.9 μM) as compared to resveratrol (IC_{50} 1.1 and 1.3 μM for COX-1 and COX-2, respectively). Results from earlier studies (*7*), using O_2 consumption as the endpoint for measuring inhibition, showed resveratrol to inhibit the hydroperoxidase activity of COX-1 and -2 with IC_{50} values of 3.7 μM and 85 μM, respectively. In the present study the endpoint was inhibition of prostaglandin production. Inhibition of synthesis progtaglandins (PGs) is a possible mechanism for cancer chemoprevention, as PGs enhance growth of malignant cells by increasing cell proliferation (*30*).

It has been suggested that cancer-inducing damage to cells might be prevented by antioxidants through the scavenging of excess free radicals that are byproducts of many normal metabolic functions, or by reducing the adverse effects of carcinogens or radiation (*31*). Thus, to further evaluate the cancer chemopreventive potential of pterostilbene, its free radical scavenging property was investigated. Preliminary information on the antioxidant activity of pterostilbene was gathered from its inhibition of the oxidation of methyl linoleate (*19*), but this test was not a direct measure of its free radical scavenging activity. Pterostilbene was tested together with resveratrol for direct radical scavenging capacity through inhibition of ABAP-derived peroxyl radicals. This test allows the definition of a TRAP index, relative to Trolox (*25*). Trolox is a reference inhibitor that removes two radicals per molecule (*32*). As illustrated in **Figure 3**, induction times with pterostilbene and resveratrol were proportional to the test concentrations. The numbers of radicals (*N*) removed per molecule of pterostilbene and resveratrol are summarized in **Table 1**. Both compounds were more effective than Trolox as free radical scavengers, and pterostilbene was as effective as resveratrol in this system. These results parallel those obtained by Stivala et al. (*12*) who found pterostilbene (EC_{50} 30.1 ± 2.1 μM) to have activity comparable to that of resveratrol (EC_{50} 24.5 ± 1.5 μM) in DPPH radical scavenging assay.

The antioxidant activity of pterostilbene and resveratrol was also evaluated using a plant system. Inhibitors of protoporphyrinogen oxidase, such as the herbicide acifluorfen, cause oxidative damage to plant cells through accumulation of the photodynamic compound protoporphyrin IX (*33*). Oxidative damage was initiated when tissue that had been incubated in darkness with the herbicide was exposed to light. Protoporphyrin

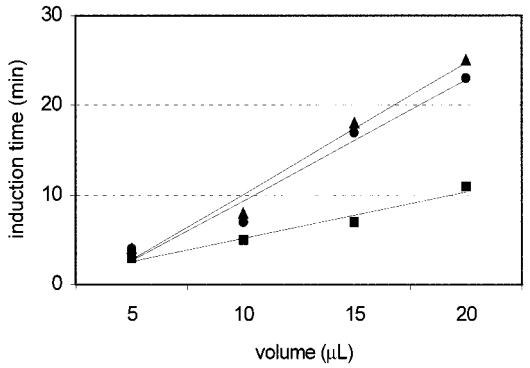

Figure 3. Induction time (min) as a function of the concentration of Trolox (150 μM initial concentration) (■), pterostilbene (●), and resveratrol (▲) (150 μM initial concentration). Induction times determined as the period during which luminescence remained reduced after addition of the samples. Values are the means of two determinations.

Table 1. Total Reactive Antioxidant Potential (TRAP) Expressed in μM Trolox Equivalents, and Number of Radicals (*N*) Trapped Per Molecule of Additive

sample	TRAP (μM)[a]	*N*
Trolox	144 ± 16	2
pterostilbene	237 ± 58	3.16
resveratrol	253 ± 53	3.37

[a] Average values, measured in the volume range of 5–20 μL, starting with initial solution at a concentration of 150 μM.

IX accumulates harmlessly in darkness, but generates singlet oxygen when exposed to light. In turn, membrane lipids are rapidly peroxidized, resulting in plasma membrane dysfunction. This effect is easily monitored by measuring electrolyte leakage from plant tissues treated with herbicide (*26*). In previous similar studies, the antioxidant α-tocopherol was shown to protect cucumber tissue from this type of oxidative damage (*27*). Employing this model, we found that resveratrol (**Figure 4A**) and pterostilbene (**Figure 4B**) gave similar results at approximately equimolar concentrations, reducing the oxidative damage by 30−50%, depending on the point in the time course. Both compounds had antioxidative activity approximately equal to that of α-tocopherol. Results obtained in this assay are in agreement with that obtained from the assay using ABAP, confirming that the antioxidant activity of pterostilbene is similar to that of resveratrol. Additionally, this assay provided clues as to the physiological function of pterostilbene and resveratrol in plants other than as phytoalexins. Furthermore, the results can be extrapolated to animal systems, but taking into consideration that the mechanisms of antioxidant activity are independent of tissue type.

The powerful antioxidant properties of resveratrol (*34*) and its ability to modulate redox-sensitive cell signaling pathways (*35*) may relate to the multitude of biological effects exhibited by this compound. Pterostilbene was demonstrated in these studies to have antioxidant activity similar to that of resveratrol, and it may also exert many biological effects similar to resveratrol. In fact, Stivala and co-workers (*12*) found that among the derivatives they tested, only pterostilbene inhibited the growth of normal fibroblasts in a dose-dependent manner similar to resveratrol (IC_{50} 60 μM for both), and pterostilbene also caused cell cycle imbalance in a manner similar to that of resveratrol. Furthermore, only pterostilbene inhibited DNA synthesis in an extent similar to resveratrol. The presence of

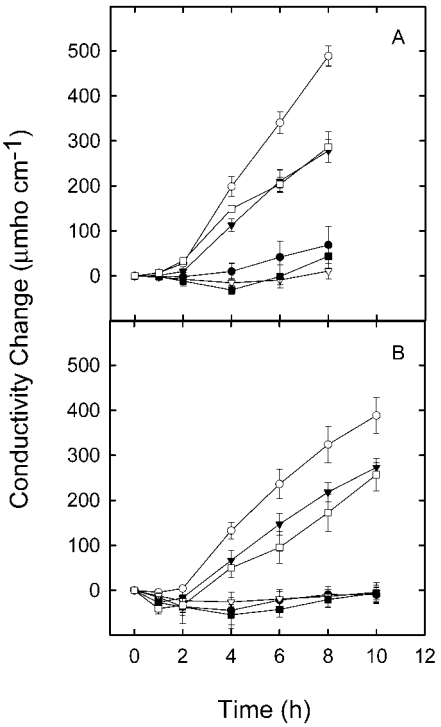

Figure 4. Effects of antioxidants on aciflourfen-caused, photooxidative cellular leakage in cucumber cotyledon disks. Control (●), 50 μM acifluorfen (○), 60 μg/mL α-tocopherol (▽), 50 μM acifluorfen + 60 μg/mL α-tocopherol (▼), 30 μg/mL resveratrol (■, panel A), 30 μg/mL resveratrol + 50 μM acifluorfen (□, panel A), 30 μg/mL pterostilbene (■, panel B), 30 μg/mL pterostilbene + 50 μM acifluorfen (□, panel B).

the 4′-hydroxyl group in both compounds was determined to be required for the antiproliferative activity.

Results reported herein indicate that pterostilbene effectively scavenges peroxyl radicals (ROO•) and reduces singlet-oxygen-induced peroxidation at levels similar to those of resveratrol. These data suggest that the antioxidant activity observed could be responsible, in part, for its capability to reduce mouse mammary gland culture carcinogenesis, and mediation of inflammatory processes shown by its weak inhibition of COX activity is possibly a less important mechanism for its chemopreventive activity. Reported cytotoxic activity of pterostilbene may also play a role in its chemopreventive property, but further investigations are needed to examine in more depth the mechanisms underlying its apparent cancer chemopreventive activity. Studies to establish in vivo efficacy would also be of value. This is the first report to demonstrate the antioxidant activity of pterostilbene, as well as resveratrol, using a plant system, and further studies to determine the physiological function of these stilbenes in the plant are warranted.

ABBREVIATIONS USED

COX, cyclooxygenase; DMBA, 7,12-dimethylbenz[*a*]anthracene; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; LDL, low-density lipoprotein; MMOC, mouse mammary gland organ culture; NMR, nuclear magnetic resonance; TRAP, total reactive antioxidant potential; Trolox, 6-hydroxy-2,5,7,8-tetramethoxylchroman-2-carboxylic acid; ABAP, 2,2′-azo-bis(2-amidinopropane).

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