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Antioxidant Effects of 2,3,6-tribromo-4,5-dihydroxybenzyl Methyl Ether (TDB) from the Red Alga, *Symphyocladia latiuscula*

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2,3,6-Tribromo-4,5-dihydroxybenzyl methyl ether (TDB) from the methanolic extract of the red alga *Symphyocladia latiuscula* exhibits major antioxidant activity. In this study, the activity of TDB against oxidative damage in deoxyribose and DNA was investigated *in vitro* for potential applications in preventing mutagenesis caused by DNA damage. TDB inhibited the oxidation of deoxyribose at concentrations of up to 1 μg/mL in the presence of Fe^{+3}-EDTA/H_2O_2. Furthermore, TDB showed no prooxidant activity as determined by absence of the reduction of bleomycin-Fe^{+3} to bleomycin-Fe^{+2}, which leads to DNA damage. Based on these results, TDB demonstrated considerable antioxidant activity without prooxidant properties.

Key words: 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether (TDB), *Symphyocladia latiuscula*, Antioxidant, Bleomycin assay

Introduction

Free radicals and active oxygen species play a pivotal role in the pathogenesis of human diseases, including cancer, aging, inflammation, diabetes, and atherosclerosis (Halliwell et al., 1992). The physiological defense systems that counteract free radicals include several endogenous enzyme systems such as catalase, glutathione reductase, and superoxide dismutase, as well as glutathione, coenzyme Q, and urate, and several exogenous factors (vitamin C, vitamin E, and phytochemicals). Natural antioxidants, such as L-ascorbic acid, tocopherol, and several flavonoids, have been shown to inhibit lipid peroxidation (Ratty and Das, 1988) by scavenging free radicals and active oxygen species (Hanasaki et al., 1994), chelating iron ions (Morel et al., 1989), and inactivating lipoxygenase (Ratty et al., 1988). However, several *in vitro* studies have demonstrated that in the presence of transition metals, many flavonoids (quercetin, catechin, mirycetin, epicatechin) can also display prooxidant activity on biological targets (Laughton et al., 1989; Heim et al., 2002). These deleterious properties might arise from the formation of reactive radicals and consequent covalent modification of biomolecules such as DNA, proteins, and carbohydrates (Kozekov et al., 2003). The biological implications of pro-oxidation are important, as illustrated by the *in vitro* characterization of the mutagenic effects of some flavonoids (Sahu and Gray, 1993). The inhibition of transition metal-induced prooxidant activity would result in major benefits in systems that contain flavonoids or any other antioxidants (Cao et al., 1997).

Symphyocladia latiuscula (Harvey) Yamada is a member of the Rhodomelaceae family in the order Ceramiales. 2,3,6-Tribromo-4,5-dihydroxybenzyl methyl ether (TDB) was isolated as one of the principal active components in the methanolic extract of this alga together with the inactive component cholesterol (Park et al., 1999; Choi et al., 2000). In previous studies (Park et al., 1999; Choi et al., 2000; Chung et al., 2001), TDB was found to exhibit significant anti-oxidative activity on the radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH), the inhibition of reactive oxygen species (ROS) generation, and the scavenging activity of peroxynitrite. TDB is a natural compound that has attracted considerable interest as a potential antioxidant additive in foods. Although the antioxidative properties of TDB have been highlighted previously, its prooxidant capability in conjunction with DNA and carbohydrates was not known. The purpose of this study was to investigate the activity of TDB

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against oxidative damage in deoxyribose and DNA and to assess any prooxidant activity.

Materials and Methods

Chemicals and material

DNA (Type XV, calf thymus), bleomycin sulfate, thiobarbituric acid, ferrous chloride, and deoxyribose were obtained from Sigma (St. Louis, MO). All other chemicals were of the highest quality available.

Leafy thalli of *Symphyocladia latiuscula* were collected from Chungsapo, Busan, Korea, in January 1998, and authenticated by Prof. K.W. Nam of the Department of Marine Biology, Pukyong National University. A voucher specimen (No. 980128) has been deposited in the author's laboratory (J.S. Choi).

Extraction, fractionation, and isolation

Powdered seaweed tissues (580 g dry weight) were refluxed with methanol for 3 h (9 L×3). The extract was concentrated to dryness *in vacuo* at 40°C to render the methanol (MeOH) extract (148 g), which was then suspended in distilled water and partitioned successively with *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH). In previous work, the CH_2Cl_2 fraction had exhibited the highest antioxidant activity against DPPH radicals. Therefore, the CH_2Cl_2 fraction (21 g) was applied to a silica-gel column and eluted with CH_2Cl_2-MeOH (gradient) to yield 22 subfractions. Of these, subfractions 5 through 9 (5.76 g) were further separated by silica-gel column chromatography with a mobile phase consisting of CH_2Cl_2:MeOH (10:1, v/v) to yield 2.282 g of TDB. The structure of TDB was confirmed by instrumental analyses (Fig. 1, Park et al., 1999).

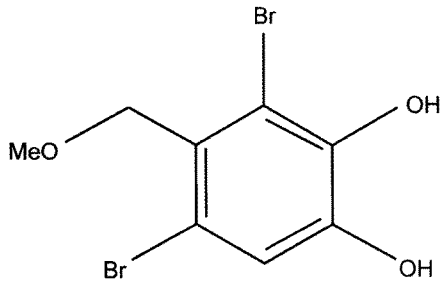

Fig. 1. Structure of 2,3,6-tribromo-4,5-dihydroybenyl methyl ether (TDB).

Deoxyribose assay

The Fenton reaction model system, which consisted of ethylenediaminetetraacetic acid (EDTA)-complexed $FeCl_3$ and hydrogen peroxide (H_2O_2) (Smith et al., 1992), was used to test the efficacy of TDB against accelerated oxidative damage of deoxyribose. The reaction mixture (3.5 mL), which contained TDB (0 to 1.0 mg/mL), deoxyribose (3 mM), H_2O_2 (1 mM), KH_2PO_4-K_2HPO_4 buffer (20 mM, pH 7.4), $FeCl_3$ (50 μM), and EDTA (100 μM), was incubated with or without additional ascorbic acid (100 μM) at 37°C for 1 h. Solutions of $FeCl_3$ and ascorbic acid were made immediately before use. The reaction was stopped by adding a trichloroacetic acid (TCA) solution and heating in a water bath at 100°C for 20 min. The extent of deoxyribose degradation was measured spectrophotometrically by monitoring the absorbance at 532 nm. The control sample lacked both TDB and ascorbic acid. As positive controls, deoxyribose degradation was monitored in the presence of gallic acid and mannitol at concentrations of up to 1 mg/mL.

Bleomycin-dependent DNA damage

The bleomycin assay described by Gutteridge et al. (1981) was modified as described by Aeschlach et al. (1994) and Chan et al. (1996). The reaction mixtures in a final volume of 0.4 mL contained 0.5 mg/mL DNA, 0.05 mg/mL bleomycin, 5.0 mM $MgCl_2$, 50 μM $FeCl_3$, and the test sample at concentrations ranging from 0 to 150 μg/mL. L-Ascorbic acid was used as a positive control and evaluated at concentrations of 0 to 150 μg/mL. All compounds and chemicals were dissolved in 30 mM KH_2PO_4-KOH buffer (pH 7.0). The reaction mixture was incubated at 37°C for 1 h. After incubation, 0.04 mL of 0.1 M EDTA was added to stop the reaction (the iron-EDTA complex is nonreactive in the bleomycin assay). Color was developed by adding 0.4 mL of 1% (w/v) thiobarbituric acid (TBA) solution dissolved in 0.05 M NaOH and 0.4 mL of 25% (v/v) HCl, followed by heating at 80°C for 10 min. After centrifugation, the extent of DNA damage was determined based on the relative increase in solution absorbance at 532 nm.

Results and Discussion

The effect of TDB on deoxyribose damage

The effect of TDB on the degree of oxidative damage of deoxyribose in the Fe^{+3}-EDTA/H_2O_2 system is shown Fig. 2. TDB suppressed the oxidative damage of deoxyribose at concentrations of up to 0.2 μg/mL, while its prooxidant activity was similar to that of gallic acid and mannitol. Gallic acid and mannitol scavenge hypochlorous acid and superoxide and inhibit the peroxidation of phospholipids. Gallic

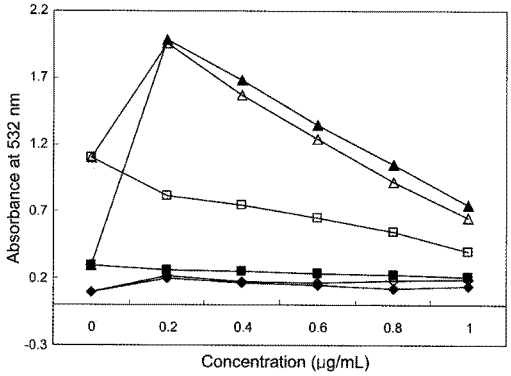

Fig. 2. Effect of TDB on deoxyribose damage in Fe^{+3}-EDTA/H_2O_2-induced oxidation system.
◆, TDB; ◇, TDB+L-ascorbic acid; ▲, Gallic acid; △, Gallic acid+L-ascorbic acid; ■, Mannitol; □, Mannitol+L-ascorbic acid.

acid may degrade the 8-hydroxy-2'-deoxyguanosine formed by its potent reducing activity (Yoshino et al., 2002). Mannitol is well known for its antioxidant activity but does not possess prooxidant properties. Therefore, TDB can be concluded to have no prooxidant activity.

Antioxidants that protect against lipid peroxidation can also cause damage to DNA, proteins, and carbohydrates. The bleomycin assay system assesses the ability of such antioxidants to damage DNA. In contrast, the deoxyribose method evaluates the degree to which such antioxidants can damage carbohydrates. The sugar deoxyribose, upon exposure to hydroxyl radicals generated by the Fenton reaction, fragments and generates a pink chromophore upon heating with TBA at low pH (Halliwell et al., 1987). The antioxidant effect of TDB is closely related to its activity as a hydrogen donor (Chung et al., 2001). TDB also inhibits lipid peroxidation by donating hydrogen to the peroxy radical as a chain-reaction intermediate. However, TDB has exhibited cytotoxicity toward MCF-7 human breast cancer cells at high concentrations (Lee et al., 2007). The current study has demonstrated that the cytotoxicity of TDB is most likely due to its antioxidant activity, as opposed to any prooxidant effect.

The effect of TDB on DNA damage

Fig. 3 shows the effect of TDB on the degree of DNA damage in the bleomycin-Fe^{+3} assay system. L-Ascorbic acid accelerated DNA damage in a dose-dependent manner. The prooxidant effect of ascorbic acid in this assay system has been previously reported (Aruoma et al., 1993) and L-ascorbic acid has been widely used for determining prooxidant activity. As shown in Fig. 3, TDB exhibited no prooxidant activity at concentrations of up to 150 ug/mL.

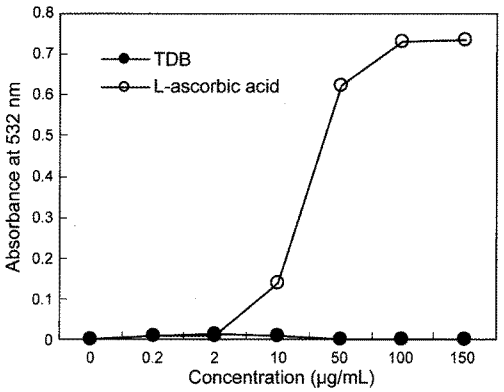

Fig. 3. Pro-oxidant activity of TDB and L-ascorbic acid on bleomycin-Fe^{+3}-induced assay system.

The bleomycin assay has also been adapted to assess the prooxidant effects of proposed food antioxidants (Aruoma et al., 1991). Bleomycin, an antitumor antibiotic, binds to DNA, causing single-strand cleavage, and degrades deoxyribose in the presence of O_2 and Fe^{+2}. DNA damage in the presence of the bleomycin-Fe complex has been adopted as a sensitive and specific method to examine potential prooxidant agents. Any compound able to reduce the bleomycin-Fe^{+3} complex to bleomycin-Fe^{+2} will stimulate DNA damage in the assay system, resulting in a positive value for prooxidant action. DNA degradation is accompanied by the formation of a product similar to MDA (malondialdehyde). Such degradation can be measured almost quantitatively by the TBA test (Gutteridge et al., 1981).

Superoxide and hydroxy radicals are the two most common free radicals in cellular oxidation reactions. The superoxide radical is normally formed first and its effects can be magnified through the production of other cell-damaging free radicals and oxidizing agents (Singh, 1989). However, the deleterious action of the hydroxy radical is the strongest among free radicals. Both superoxide and hydroxy radicals actively participate in the initiation of lipid peroxidation. Oxidation of unsaturated fatty acids in biological membranes leads to lipid oxidation and eventual destruction of membrane lipids, which produce breakdown products such as malondialdehyde. Anti-oxidative compounds in living systems are classified as preventive or chain-breaking antioxidants (Halliwell et al., 1990). Although vitamin C

is a strong antioxidant, a high intake of vitamin C may lead to increased prooxidant activity when free transition metals are also available. ROS produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions, and metabolic processes have a wide variety of pathological effects, such as DNA damage, carcinogenesis, and cellular degeneration related to aging (Aruoma, 1991). Antioxidants have an important role in the suppression of free radical production in the cellular environment and in the prevention of diseases associated with aging. For these reasons, natural antioxidants without prooxidant activity have attracted great interest from the nutraceutical and pharmaceutical fields. In the current study, TDB was confirmed as a natural antioxidant without prooxidant activity in the bleomycin-Fe^{+3}-induced assay system. Moreover, TDB demonstrated potential as an antioxidant ingredient in health-functional food products.

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