Antioxidative Activities of Natural Compounds Found in Plants

Takashi Miyake and Takayuki Shibamoto*

Department of Environmental Toxicology, University of California, Davis, California 95616

The antioxidative activity of natural plant products—lacinilene A, naringin, galangin, and rutin—was examined using lipid peroxidation systems consisting of either ethyl linoleate, ethyl linolenate, or ethyl arachidonate plus Fenton's reagent. Inhibitory activity of plant products toward malonaldehyde (MA) formation from lipids was measured using gas chromatography. Lacinilene A, which showed the strongest antioxidative acitivity among the chemicals tested, inhibited MA formation from ethyl linolenate and ethyl arachidonate by 100% at the levels of 3.0 and 0.5 μmol, respectively. Natural flavonoid compounds naringin, galangin, and rutin exhibited appreciable antioxidative activities at doses lower than 0.125 μmol. Rutin, which exhibited the strongest activity among the three flavonoids, inhibited MA formation from ethyl arachidonate by 70% at the level of 0.125 μmol. These flavonoids exhibited only slight inhibition of MA formation at levels higher than 0.5 μmol from the ethyl esters of the three fatty acids.

Keywords: *Antioxidants; lipid peroxidation; malonaldehyde; plant components*

INTRODUCTION

Since some synthetic antioxidants, such as butylated hydroxyanisol, have been found to be toxic to experimental animals (Ford et al., 1980), natural plant products began to receive much attention as sources of safe antioxidants (Frankel et al., 1996). The main source of natural antioxidants is various kinds of plant products such as oil, seeds, cereals, beans, and nuts (Namiki, 1990). Because plant seeds must retain germination ability for long-term preservation, they usually contain effective antioxidants in or around their germs. Also, plant leaves such as tea leaves and barley leaves contain strong antioxidants (Osawa et al., 1992). For example, an isoflavonoid isolated from young barley leaves inhibited malonaldehyde formation from squalene by almost 100% upon UV irradiation at the level of 10 μmol/mmol (Nishiyama et al., 1993).

Lipid peroxidation initiated by reactive radicals, such as the hydroxy radical, causes many biological damages, including atherosclerosis (Iwakami, 1965), liver diseases (Suematsu and Abe, 1982), diabetes (Saito et al., 1979), and aging (Sagai and Ichinose, 1980). Moreover, the formation of lipid peroxides and their secondary products such as reactive carbonyl compounds also causes various kinds of biological damage. In particular, the aldehydic products malonaldehyde (MA), formaldehyde, acetaldehyde, and 4-hydroxynonenal are known to be mutagenic and carcinogenic (Marnett et al., 1985; Feinman, 1988; Esterbauer et al., 1991; Chaudhary et al., 1994). These effects may be the result of covalent linkages formed when the electrophilic carbonyl reacts with nucleophilic amino and sulfhydryl groups of proteins and DNA.

There is a pressing need to find safe, natural antioxidants, not only to prevent oxidative deterioration in foods but also to inhibit oxidative damages caused by lipid peroxidation. Therefore, in the present study, natural compounds found in plants were tested for antioxidative activity using a lipid peroxidation system. Among the natural products tested, lacinilene A exhibited the most potent antioxidative activity.

* Author to whom correspondence should be addressed [telephone, (916) 752-4523; fax, (916) 752-3394; e-mail, tshibamoto@ucdavis.edu].

MATERIALS AND METHODS

Chemicals. Ethyl linoleate, ethyl linolenate, ethyl arachidonate, butylated hydroxytoluene (BHT), trizma hydrochloride, and trizma base were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Methylhydrazine (NMH), 2-methylpyrazine, naringin (4′,5,7-trihydroxylflavanone), galangin (3,5,7-trihydroxy-2-phenyl-4*H*-1-benzopyran-4-one), rutin {3-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one}, sodium dodecyl sulfate (SDS), and ferrous chloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Hydrogen peroxide was bought from Fisher Scientific Co., Ltd. (Fair Lawn, NJ). The standard stock solution of 2-methylpyrazine was prepared by adding 10 mg of 2-methylpyrazine to 1 mL of solvent and was stored at 5 °C. Authentic 1-methylprazole (1-MP) was synthesized according to the method developed by Umano et al. (1988).

2″-*O*-Glycosylisovitexin (2″-*O*-GIV). 2″-*O*-GIV was isolated from young green barley leaves (*Hordium vulgare* L. var. *nudum* Hook), harvested 2 weeks after germination, by a method reported previously (Osawa et al., 1992).

Lacinilene A (6-Norcadalen-7-ol). Lacinilene A was synthesized by a method reported previously (Tanaka et al., 1992).

Oxidation of Ethyl Linoleate, Ethyl Linolenate, and Ethyl Arachidonate with Fenton's Reagent (Fe^{2+}/H_2O_2) in the Presence of the Natural Products. An aqueous solution (5 mL) containing 10 μL of ethyl linoleate, ethyl linolenate, or ethyl arachidonate, 0.25 mmol of trizma buffer (pH 7.4), 1 μmol of ferrous chloride, 0.5 μmol of hydrogen peroxide, 0.75 mmol of potassium chloride, and 0.2% of surfactant SDS was incubated with varying amounts of lacinilene A, naringin, galangin, or rutin for 16 h at 37 °C in a 20 mL test tube. The oxidation of samples was stopped by adding 50 μL of a 4% BHT solution (Ichinose et al., 1989). The sample tubes were covered with aluminum foil during incubation to avoid any influence of light on the lipid peroxidation. A known antioxidant, BHT, was used to compare antioxidative activity to that of the compounds tested.

Analysis of MA Formed from Lipids upon Oxidation. Antioxidative activity was determined by analyzing MA formed from lipids upon oxidation after derivatizing to 1-MP with NMH (Tamura et al., 1991). NMH (30 μL) was added to the above oxidized lipid solutions, and then the solutions were stirred for 1 h at 25 °C. Each reaction solution was extracted

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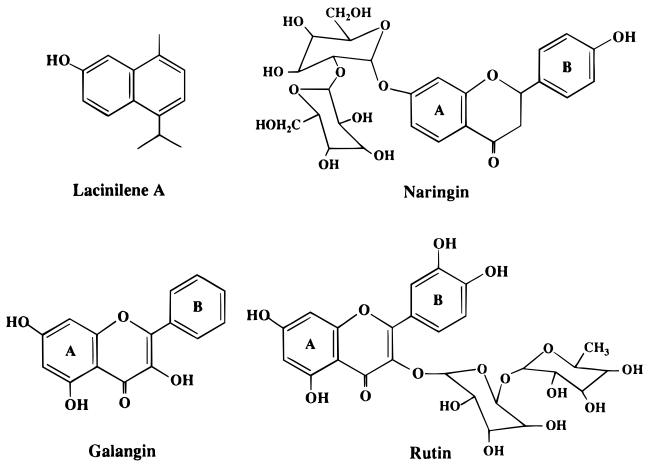

Figure 1. Structure of lacinilene A, naringin, galangin, and rutin (A = ring-A, B = ring-B).

with 10 mL of dichloromethane using a liquid−liquid continuous extractor for 3 h. The solution was saturated with NaCl prior to extraction to prevent the formation of an emulsion. The extract was adjusted to exactly 10 mL by adding dichloromethane. After 50 μL of 2-methylpyrazine solution was added as a gas chromatographic internal standard, the solution was analyzed for 1-MP by a gas chromatograph (GC) with a nitrogen−phosphorus detector (NPD).

A Hewlett-Packard (HP) Model 5890 GC equipped with an NPD and a 30 m × 0.25 mm i.d. (d_f = 1 μm) DB-WAX bonded-phase fused silica gel capillary column (J&W Scientific, Folsom, CA) was used to analyze 1-MP. The detector and injector temperatures were 250 °C. The linear velocity of helium carrier gas was 30 cm/s at a split ratio of 23:1. The oven temperature was programmed from 60 to 180 °C at 4 °C/min and held for 10 min. GC peak areas were integrated with a Tsp SP 4400 series integrator. An HP Model 5890 series II GC interfaced to an HP 5971 mass spectrometer was used to confirm the pyrazole derivative, *N*-methylpyrazole, in the sample. The GC conditions were the same as for the above GC. The mass spectra were obtained by electron impact ionization at 70 eV and an ion source temperature of 250 °C. Each experiment was repeated three times.

RESULTS AND DISCUSSION

Figure 1 shows the structures of natural products tested for antioxidative activity in the present study. All compounds contained the phenolic hydroxy group. The sesquiterpenoid lacinilene A is one of the major components of essential oils from heartwood (Suzuki et al., 1972) and sapwood (Nishikawa et al., 1972). Naringin, which is a bitter glucoside of grapefruit, was first discovered in 1857 (Robinson, 1983). Galangin is found in galanga root, *Alpininia officinarun* (Robinson, 1983). Rutin was first found in buckwheat (Robinson, 1983). All compounds except lacinilene A are flavonoid compounds.

Figures 2−7 show the results of antioxidative tests on lacinilene A, naringin, galangin, and rutin. Among the natural products tested, lacinilene A exhibited the most potent antioxidative activity (Figures 2−4). The levels of MA formed from 10 μL each of ethyl linoleate, ethyl linolenate, and ethyl arachidonate were 192.8 ± 4.3, 407.3 ± 5.5, and 676.8 ± 3.1 nmol, respectively, under the conditions used in the experiments without lacinilene A or BHT. The values are mean ± standard deviation (n = 3). MA forms from polyunsaturated fatty acids (PUFAs) in relatively large amounts. A fatty acid with a higher number of double bonds (such as arachidonic acid) produces more MA than a fatty acid with a lower number of double bonds (such as linoleic acid)

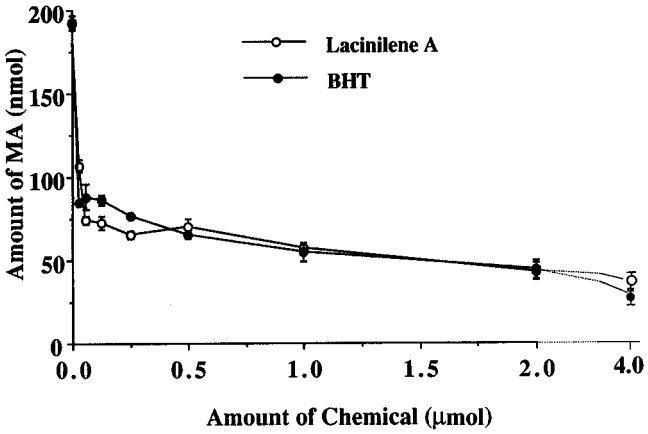

Figure 2. Effect of lacinilene A and BHT on MA formation from ethyl linoleate oxidized by Fenton's reagent. Values are the average of three replications.

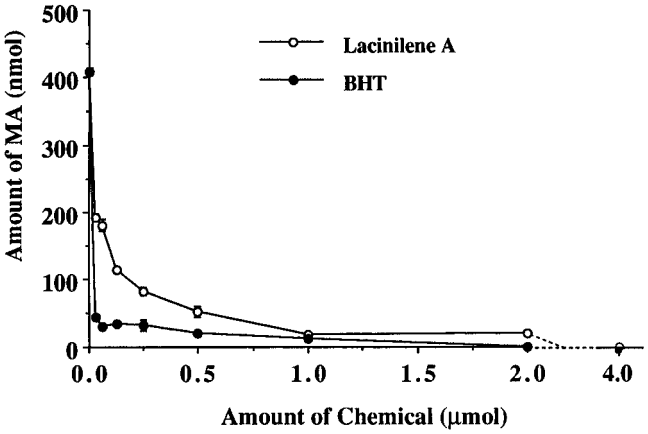

Figure 3. Effect of lacinilene A and BHT on MA formation from ethyl linolenate oxidized by Fenton's reagent. Values are the average of three replications.

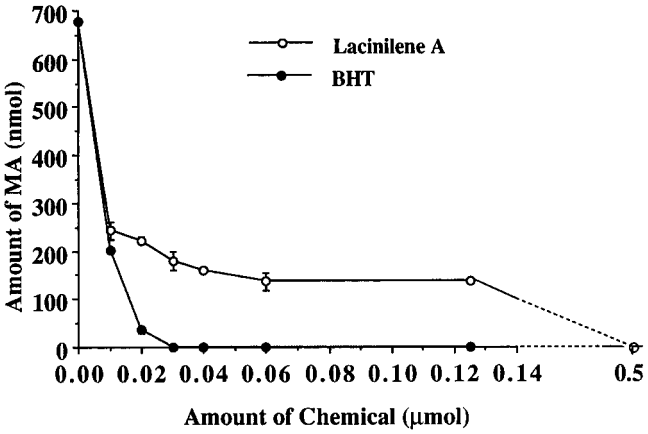

Figure 4. Effect of lacinilene A and BHT on MA formation from ethyl arachidonate oxidized by Fenton's reagent. Values are the average of three replications.

(Tamura et al., 1991). The results obtained in the present study are consistent with the previous study. Lacinilene A and BHT exhibited similar inhibitory activity of MA formation from ethyl linoleate (Figure 2). They inhibited MA formation by nearly 70% at the level of 2.0 μmol. Lacinilene A inhibited MA formation from ethyl linolenate by more than 90% at the level of 1.0 μmol and by 100% at the level of 3.0 μmol. In the case of ethyl arachidonate, BHT inhibited MA formation by 100% at the level of 0.03 μmol, whereas lacinilene A inhibited MA formation by 75% at the same level. The

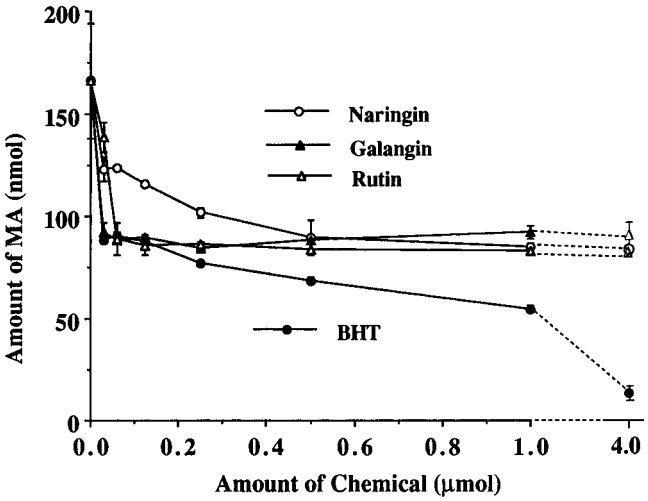

Figure 5. Effect of naringin, galangin, rutin, and BHT on MA formation from ethyl linoleate oxidized by Fenton's reagent. Values are the average of three replications.

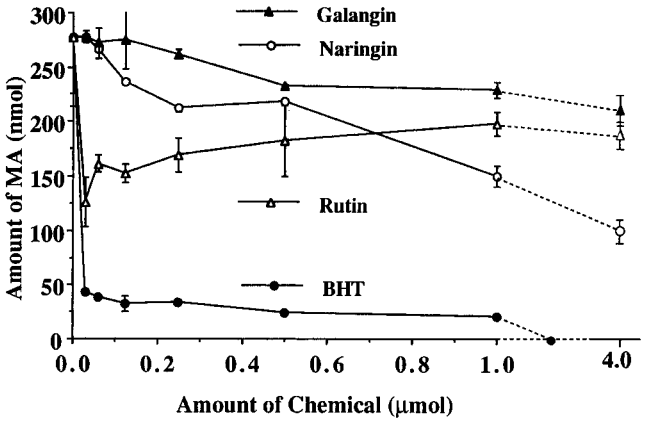

Figure 6. Effect of naringin, galangin, rutin, and BHT on MA formation from ethyl linolenate oxidized by Fenton's reagent. Values are the average of three replications.

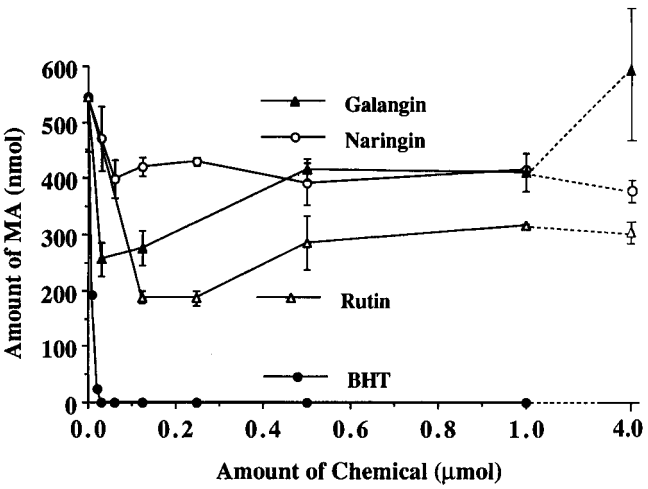

Figure 7. Effect of naringin, galangin, rutin, and BHT on MA formation from ethyl arachidonate oxidized by Fenton's reagent. Values are the average of three replications.

greatest inhibition was obtained by lacinilene A at the level of 0.5 μmol.

The natural flavonoid compounds of naringin, galangin, and rutin exhibited certain appreciable antioxidative activities at doses lower than 0.125 μmol (Figures 5−7). In particular, rutin inhibited MA formation from ethyl arachidonate by 70% at the level of 0.125 μmol (Figure 7). In the case of ethyl linoleate, all three flavonoids exhibited dose-related activities and inhibited MA formation by nearly 30% at a dose of 0.5 μmol, at which point their activities leveled off. However, in the case of ethyl arachidonate, MA formation from ethyl arachidonate increased when the dose of galangin increased over 1.0 μmol (Figure 7). It is difficult to explain this phenomenon. Some prooxidants may be produced in this sample.

Flavonoids occur throughout the entire plant kingdom from fungi to angiosperms. Numerous flavonoids have been characterized, and new structures are being reported at an increasing rate. The most widely distributed flavonoids, flavones and flavonols, are mainly hydroxylated in the B-ring at the 3′- and 4′- positions (rutin) followed by the 4′-position only (naringin). Many of the flavonoids and related compounds are known to possess strong antioxidative characteristics (Pratt, 1979; Dziedzic and Hudson, 1983). The major mode of flavonoids as antioxidants is in their ability to scavenge free radicals, such as the hydroxy radical, deriving from the phenolic moiety of the structure (Pratt, 1976). Hydroxylation of the B-ring is an important factor for the antioxidative activity of these compounds, although it is not a prerequisite for manifesting the activity (Pratt, 1976; Siegers et al., 1982). In the present study, rutin, which has hydroxyl groups at the 3′- and 4′-positions of the B-ring, exhibited the highest antioxidative activity among the three flavonoids tested. Naringin, which possesses a single hydroxy group at the 4′- position of the B-ring, exhibited slight antioxidative activity. On the other hand, galangin, which does not possess a hydroxy group in the B-ring, exhibited the lowest antioxidative activity among the three flavonoids tested.

The relationship between lipid peroxidation and diseases such as cancer is complex and not yet well understood. However, lipid peroxidation may play a role in the promotional phase of carcinogenesis *via* the reaction of the secondary products with lipid membranes, proteins, and DNA. Among the toxic secondary products of lipid peroxidation, MA is one of the most studied products because of its extreme reactivity arising from its bifunctionality. DNA reacts with MA to form fluorescent products that have been reported to correlate with the loss of DNA template activity (Reiss et al., 1972). Also, MA is reportedly mutagenic (Basu et al., 1988) and a suspected carcinogen (Shamberger et al., 1974). Inhibition of lipid peroxidation with natural products may prevent occurrence of certain diseases. The antioxidative activity of the natural products exhibited in the present study is not as strong as that of the known antioxidant BHT. However, these products, in particular, the flavonoids, are present in natural plant foods such as cereals and beans in relatively large amounts (Ramarathnam et al., 1989; Hayes et al., 1977).

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